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# THE NON-ENZYMIC REDUCTION OF TRIMETHYLAMINE OXIDE TO TRIMETHYLAMINE, DIMETHYLAMINE, AND FORMALDEHYDE[1]

BY E. B. VAISEY[2]

## Abstract

Trimethylamine oxide at temperatures between 22°–24° C. is readily reduced by cysteine in the presence of iron or hemoglobin as a catalyst. Trimethylamine is the major product of this reduction, but appreciable amounts of dimethylamine and formaldehyde are also formed. The maximum reduction of trimethylamine oxide is between pH 7 and 8. Glutathione could not replace cysteine as a reducing agent, neither could copper or manganese replace iron compounds as catalysts. Larger amounts of iron ($Fe^{++}$) could also reduce trimethylamine oxide with the formation of the above products. A mechanism for the reaction is proposed.

## Introduction

Dimethylamine (DMA) and trimethylamine (TMA) in certain dead marine fishes arise mostly as a result of bacterial activity; for this reason the determination of these compounds has been used as a spoilage index (2, 3, 16, 17). Although it is well known that the precursor of TMA is trimethylamine oxide (TMAO) (3), the precursor of DMA has not been discovered.

TMAO is decomposed into TMA, DMA, and formaldehyde by heating above 100° C. (9). Hydrolysis of the sulphur dioxide salt of TMAO resulted in the formation of similar products (14). Reay and Shewan (15) reported that bacteria, able to produce DMA in fish muscle juice, failed to produce it in a synthetic medium also containing the oxide. We have observed that washed cell suspensions of *Serratia marcescens* produced only TMA during the reduction of TMAO in media buffered at pH 6.5, 7.0, and 7.5, containing glucose as a hydrogen donator.

However, since TMAO was the precursor of DMA in the above chemical experiments, it was decided to investigate the possibility of reducing TMAO by chemical means, under conditions similar to those of the biological experiments reported above. Ackermann *et al.* (1) reported the reduction of TMAO by cysteine. Moreover cysteine can only reduce molecular oxygen in the presence of certain metals (4). The reduction of TMAO by cysteine in the presence of iron has therefore been more carefully studied.

[1]*Manuscript received June 21, 1956.*
*Contribution from the Fisheries Research Board of Canada, Technological Station, Halifax, Nova Scotia.*
[2]*Present address: Oregon State College, Science Research Institute, Corvallis, Oregon, U.S.A.*

## Materials

*Inorganic chemicals.*—Reagent grade or better.

*Trimethylamine hydrochloride.*—Reagent grade trimethylamine hydrochloride is generally contaminated with dimethylamine. The latter was removed by treating a solution of the amine hydrochloride with a solution of sodium nitrite at room temperature, and extracting the nitrosamines thus formed with ether. The aqueous solution was then made strongly alkaline and distilled into concentrated hydrochloric acid. The trimethylamine hydrochloride crystals obtained after evaporation of the acid mixture were recrystallized twice from ethanol. The product (100 mgm. sample) was found to be free of dimethylamine when tested by the method given below.

*Trimethylamine oxide dihydrate.*—Trimethylamine hydrochloride, prepared as above, was made strongly alkaline and distilled into cold 3% hydrogen peroxide (9). When the peroxide mixture had stood for 24 hr. at room temperature, a few small pieces of clean sheet platinum were added to catalyze the decomposition of the excess peroxide. After standing a further 24 hr., the platinum was removed and the mixture was concentrated by distillation at 65° C. under reduced pressure. The trimethylamine oxide crystals so obtained were recrystallized once from ethanol and twice from ethanol–acetone. The product melted at 96° C. as reported for trimethylamine oxide dihydrate (8), and a 100 mgm. sample was found to be free of hydrogen peroxide when tested by the very sensitive method of Denigès (6).

*Hemoglobin.*—A 1% solution of hemoglobin (Difco) was dialyzed overnight against distilled water.

L-*Cysteine hydrochloride.*—Nutritional Biochemical Corporation.

## Methods

The reactions were performed in 15-mm. test tubes at room temperature (22°–24° C.). The reaction solutions generally contained TMAO, cysteine, catalyst, and buffer as indicated under Figs. 1, 2, or 3. Phosphate buffer seriously interfered with the reactions. Substitution of veronal buffers was satisfactory if the solution of cysteine was previously adjusted to the pH of the buffer. Anaerobic techniques were not adopted in these experiments since under nitrogen the yield only increased by about 6%.

The reaction was stopped when desired by precipitating cysteine with a slight measured excess of cadmium acetate or by precipitating hemoglobin when present with trichloroacetic acid added to establish a concentration of 5%. After centrifugation, the volatile amines were removed from an aliquot of the supernatant fluid by the Conway technique of Beatty and Gibbons (3) after diffusion for two hours at 37° C. The resultant acid mixture was made up to

TMAO to TMA and a 0.25% reduction of TMAO to DMA, under the conditions of these experiments. The specificity of assay for TMA and DMA was established under the conditions of the experiments.

## Experimental

Fig. 1 shows the rate at which TMA and DMA are formed during the reduction of TMAO with iron and hemoglobin as catalysts. It is not justifiable to compare the reaction rates of the two catalysts, since the iron content of hemoglobin is 0.33%, so that the ratio of iron in the two experiments was about 6 : 100. The iron-catalyzed reaction was believed to be very near the equilibrium point when the experiment was terminated, for about this time, the violet color of the ferric–bicysteine complex disappeared.

Experiments similar to those reported in Fig. 1 but of 90 min. duration, showed that neither copper nor manganese catalyzed the reduction of TMAO by cysteine. Nor could glutathione replace cysteine as a reducing agent in the presence of any of the above inorganic compounds. These metals are known to catalyze the autoxidation of either cysteine or glutathione (4). Their failure to catalyze the reduction of TMAO was perhaps due to an

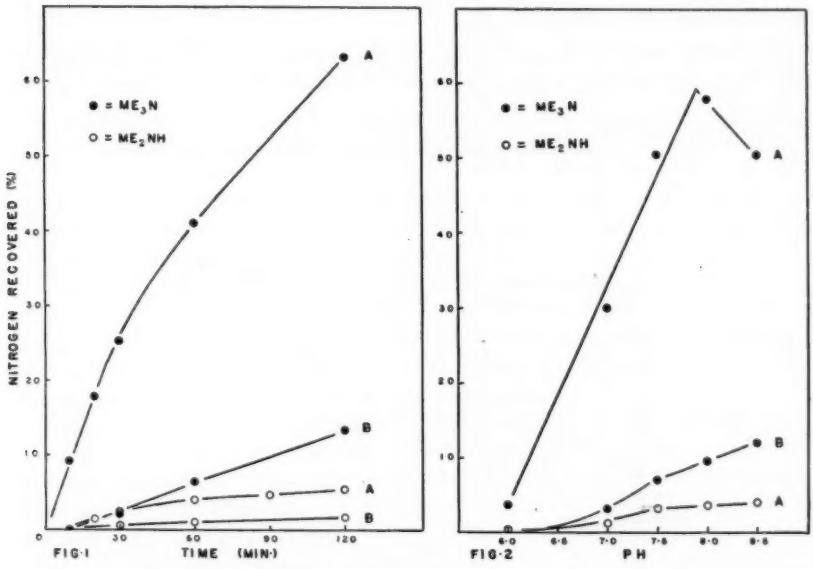


FIG. 1. TMAO reduction rate in the presence of cysteine with iron (A) and hemoglobin (B) as catalysts. Reaction mixture contained 1 ml. of 0.05 *M* TMAO, 1 ml. of 0.1 *M* cysteine, 1 mgm. of $FeCl_2 . 4H_2O$ or 20 mgm. of hemoglobin, and sufficient 0.05 *M* veronal buffer at pH 7.5 to give a final volume of 6 ml.

FIG. 2. Effect of pH on TMAO reduction rate in the presence of cysteine with iron (A) and hemoglobin (B) as catalysts. The reaction mixture was similar to that described in FIG. 1 except that 0.05 *M* phthalate buffer was used to obtain a pH of 6.0. Incubation time about 75 min.

unfavorable oxidation–reduction potential of the appropriate substrate–metal complexes. The $E_0'$ of the TMAO–TMA system is not known exactly, but Castell's work (5) would indicate that it is between +0.12 and +0.14 v. at pH 7.2.

Fig. 2 shows that the rate of reduction of TMAO with both iron and hemoglobin as catalysts is greater above pH 7. In the case of the iron-catalyzed reactions DMA formation was still increasing slightly at the termination of the experiment at pH 8.5, whereas TMA formation was maximum at about pH 8.0.

The results in Fig. 3 show that cysteine is unable to reduce TMAO without the mediation of a sufficient amount of catalyst. This was substantiated by the controls in the preceding experiments, but is contrary to the findings of Ackermann *et al.* (1). It seems probable that the cysteine used by these workers was contaminated with iron.

It may also be seen (Fig. 3) that the formation of both TMA and DMA increases with increasing iron concentration. It should be noted, however, that iron alone (curve B) is able to reduce TMAO to TMA and DMA when present in sufficient quantity. Ackermann *et al.* (1) also found that iron reduced TMAO to TMA, but they were apparently unaware that DMA was formed during the reaction.

Qualitative tests for formaldehyde, using chromotropic acid as described by Feigl (12), showed that it also was formed during the decomposition of TMAO.

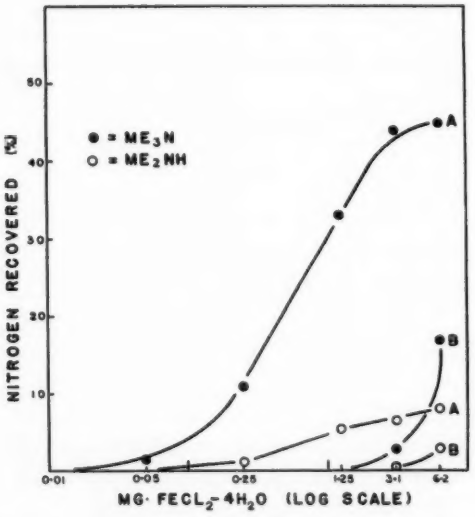


FIG. 3. Effect of various amounts of iron on TMAO reduction rate in the presence (A) and absence (B) of cysteine. The reaction mixture was similar to that described in FIG. 1. Incubation time about 45 min.

## Discussion

The mechanism for the catalyzed reduction of TMAO by cysteine perhaps can be pictured as proceeding according to the equations below, although in reality it probably proceeds with the formation of catalyst–substrate intermediates. Equations 1 and 2 respectively represent the reduction and subsequent oxidation of iron, including hemoglobin iron, which allows them to function catalytically.

$$2RSH + 2Fe^{+++} \longrightarrow RS{-}SR + 2H^{+} + 2Fe^{++} \qquad [1]$$

$$(CH_3)_3N \longrightarrow O + 2H^{+} + 2Fe^{++} \longrightarrow (CH_3)_3N + 2Fe^{+++} + HOH \qquad [2]$$

$$(CH_3)_2N(CH_2{-}H) \longrightarrow O + H^{+} \longrightarrow \left[(CH_3)_2N{=}CH_2\right]^{+} + HOH \qquad [3]$$

I

$$\left[(CH_3)_2N^{\oplus}(CH_2{-}H)\right] OH^{-} \longrightarrow (CH_3)_2N(CH_2{-}O{-}H) \longrightarrow H_2C{=}O + (CH_3)_2NH \qquad [4]$$

I II

A mechanism to account for the formation of DMA and formaldehyde is shown in equations 3 and 4 and is based on the evidence that they occur as a consequence of the reduction of TMAO. The possibility that they were formed by an intermolecular reaction between TMA and TMAO was eliminated upon failure to detect DMA in solutions buffered at pH 7 containing TMA, TMAO, and catalytic amounts of iron. A second possibility that TMAO rearranged to form O,N,N-trimethylhydroxylamine was also eliminated when it was found that Jones and Major (13) had prepared this hydroxylamine. They reported the compound as being fairly stable under ordinary conditions and that it decomposed on heating with strong hydrochloric acid to monomethylamine, methyl chloride, and formaldehyde.

Comparing the results and experimental conditions of the catalytic reductions of TMAO with the enzymic reductions mentioned in the introduction, it seems that the failure to detect DMA (15), can best be explained on the basis of enzyme specificity. If this is so, then TMAO is not likely to be the precursor of DMA in dead fish.

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# THE PURIFICATION OF SARINASE FROM BOVINE PLASMA[1]

By P. A. Adie

The purification of bovine plasma sarinase has been carried out using alcohol precipitation methods and ultracentrifugation. A protein solution was obtained which was 1250 times more active per unit weight of protein than the original plasma. Sixty per cent of this activity was lost on freeze-drying. The electrophoretic pattern of the preparation showed that the material was apparently homogeneous.

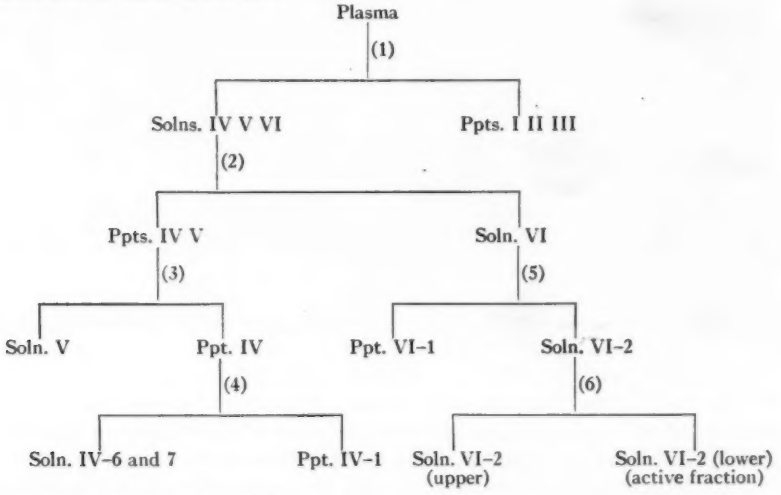
## Introduction

During the past ten years a number of reports have dealt with the enzymatic hydrolysis of organophosphorus cholinesterase inhibitors (3, 4, 8, 9). In a paper concerning the enzymatic hydrolysis of tabun (ethyl N,N-dimethylphosphoramidocyanidate) (4), Augustinsson made passing reference to the hydrolysis of sarin (isopropyl methylphosphonofluoridate). Recently this latter reaction was examined in more detail (2).

Augustinsson (4), Mazur (8), and Mounter (9) record the results of work on the purification of enzymes which hydrolyze organophosphorus compounds. The present report describes the purification of the sarin hydrolyzing enzyme (sarinase) from bovine plasma by means of the alcohol precipitation method (Cohn *et al.* (5)) and by ultracentrifugation.

## Experimental and Results

Bovine plasma, from heparinized blood, was fractionated according to Cohn's method for the preparation of human plasma (5). The procedure can be shown in outline as follows.




[1]*Manuscript received June 7, 1956.*
*Contribution from the Physiology Section, Defence Research Board, Suffield Experimental Station, Ralston, Alberta, as Suffield Technical Paper No. 90 (DRB Project No. D52-89-50-15).*

The conditions for the various separations are given in Table I.

TABLE I

CONDITIONS FOR SEPARATIONS (TEMP. = −5° C.)

| Separation number (refers to diagram) | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Conc. of metal (moles/liter) | Na (0.04) | Zn (0.02) | Zn (trace)<br>Ba (0.01)<br>Na (0.02) | Zn (0.0005)<br>Na (0.05) | Ba (0.02) | Ultracentrifuge |
| pH | 5.8 | 5.8 | 5.5 | 6.2 | 6.1 | |
| Ethanol (mole fract.) | 0.066 | 0.066 | 0.051 | 0.051 | 0.066 | |

Each fraction obtained was tested for sarinase activity and an aliquot was retained for nitrogen determinations. In order to remove the last traces of alcohol and ensure that the solutions tested were of equal ionic strength, all fractions, before being tested, were dialyzed for at least fourteen hours with internal stirring against four or more changes of physiological saline. The results are shown in Table II.

TABLE II

SPECIFIC SARINASE ACTIVITY OF FRACTIONS OF BOVINE PLASMA

| Fraction* | Specific activity†,‡ ($\mu$l.$CO_2$/mgm.N/30 min.) | Activity of fraction/ activity of plasma |
|---|---|---|
| Plasma | 3.7 | 1.00 |
| I II III | 1.4 | 0.38 |
| IV–1 | 5.3 | 1.43 |
| IV–6 and 7 | 7.6 | 2.05 |
| V | 24 | 6.49 |
| VI | 330 | 89 |
| VI–1 | 9.3 | 2.5 |
| VI–2 | 893 | 241 |

**Cohn's human plasma fraction numbering.*
*†Nitrogen determined by micro-Kjeldahl method (6).*
*‡Carbon dioxide evaluation determined manometrically (10) (substrate 0.5 ml. of 0.04 M sarin, temperature 37° C.)*

Fraction VI–2 was centrifuged for 14 hr. at 40,000 r.p.m., 105,400 × *g* in a preparative ultracentrifuge. The upper two-thirds and lower one-third were tested for sarinase activity. A small amount of precipitate which adhered to the walls of the tubes was not tested. The lower fraction had a specific activity of 4625 $\mu$l.$CO_2$/mgm.N/30 min., which is 1250 times the activity of the original plasma. The upper fraction exhibited no sarinase activity.

Fig. 1 shows the electrophoretic patterns of fractions VI and VI–2 (Table II) and fraction VI–2 (lower). The solutions were concentrated by freeze-drying to obtain these patterns.

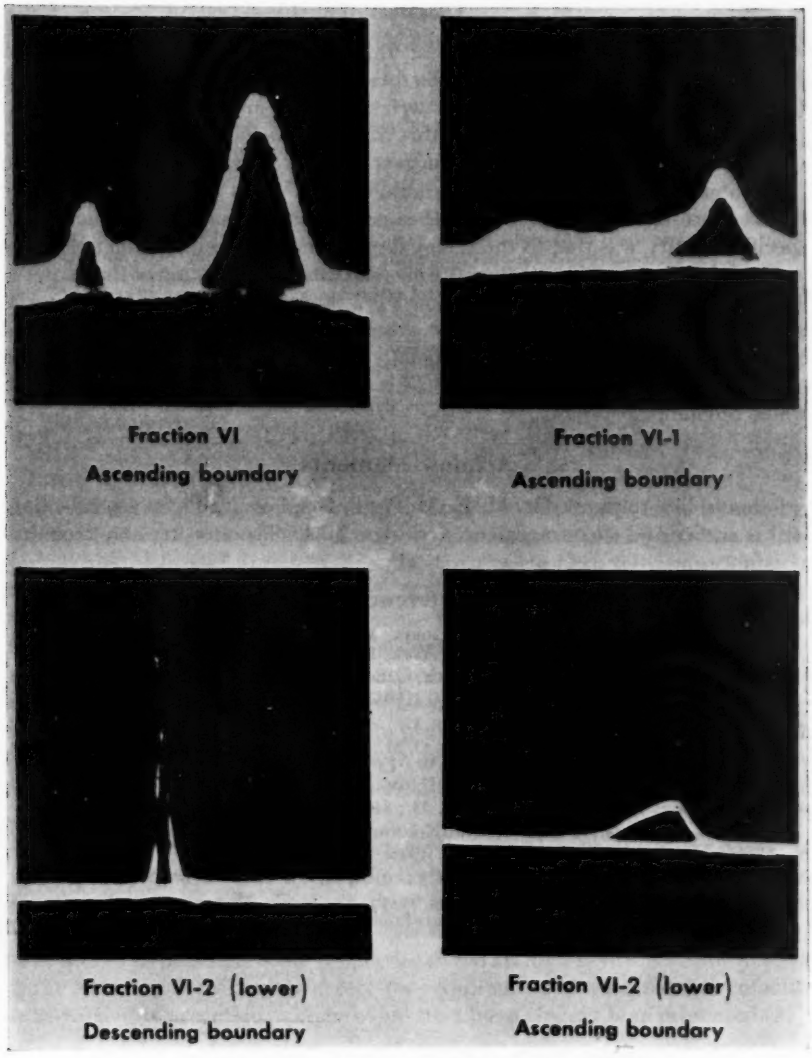



FIG. 1. Electrophoretic pattern of VI subfractions.

Dialysis of fraction VI–2 (lower), freeze-drying, and subsequent solution reduced the specific sarinase activity by 60%. This loss of activity was suffered despite the fact that the dried material (a white powder) was dissolved in physiological saline containing the following:

$Mg^{++}$ ($1\times10^{-3}$ *M*), $Ca^{++}$ ($5\times10^{-4}$ *M*), $Mn^{++}$ ($5\times10^{-5}$ *M*) (8).

## Discussion

The results obtained indicate that the sarinase activity is concentrated in Cohn's fraction VI–2. In *human* plasma this fraction contains $\alpha_1$-lipid free glycoprotein of small molecular weight, having an isoelectric point about pH 3 (5). The specific activity of this fraction was increased many times by ultracentrifugation. This would suggest that in bovine plasma more than one protein was present in bovine fraction VI–2. This was borne out by the electrophoresis pattern obtained. It is possible that much of the increase in specific activity was due to the removal of the material which adhered to the walls of the ultracentrifuge tubes as well as to the sedimentation of the sarinase. The electrophoresis pattern of fraction VI–2 (lower) showed that this material was apparently homogeneous. The sharp peak produced by the descending boundary is similar to the $\beta$ anomaly encountered with the descending boundary of human plasma (1). This phenomenon does not occur with whole bovine plasma (7).

## Acknowledgments

I should like to thank Dr. M. K. McPhail, Head of the Physiology Section, for his advice and encouragement, and Mrs. M. Dickson for her able technical assistance.

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# THE ENZYMATIC REDUCTION OF TETRAZOLIUM SALTS BY AMINES[1]

By J. R. Lagnado and T. L. Sourkes


## Abstract

An enzyme system effecting the dehydrogenation of amines has been detected in rat brain and liver suspensions by the use of tetrazolium dyes as terminal electron acceptors. Kinetic data on this system are presented and the evidence for requirement of a cofactor is described. Thus, after washing or dialysis, rat brain suspensions have a considerably lowered tetrazolium reducing activity, which can be restored by addition of boiled extracts of rat liver, rat brain, pig liver, or baker's yeast. The heat-stable cofactor in pig liver and rat brain which is necessary for the activity of the tetrazolium reducing system is dialyzable. Pig liver extracts lose their cofactor activity on ashing. The nature of the electron transporting system active in amine dehydrogenation is discussed and the properties of the tetrazolium reducing and the amine oxidase systems are compared.


Little is known of the chemical constitution of amine oxidase (2, 34). Development of our knowledge on this subject is important for enzymology, but not less so for physiology in view of the function of this oxidase in the normal metabolism of noradrenaline (24), adrenaline (25), serotonin (28), and other amines of biological origin (23, 26). Unfortunately, lack of progress in purifying the enzyme has hampered achievement of this aim and, consequently, indirect approaches to its chemical nature have had to suffice. One of these approaches has been the replacement of oxygen, as the terminal hydrogen acceptor, by dyes in the attempt to isolate parts of a possible multienzyme system; but apart from being used to provide a few data of a qualitative kind (3, 21), this technique has hardly been exploited. Recently tetrazolium dye reduction has been applied to the histochemical localization of amine oxidase (8, 10).

Preliminary attempts in this laboratory to place on a quantitative basis the effect of amines on the enzymatic reduction of tetrazolium salts led to encouraging results, and since no systematic study of this has been reported, further experiments toward this end were undertaken here. These experiments have served to illustrate some properties of the amine dehydrogenating system in rat liver and brain and to detect the requirement of a heat-stable cofactor in the anaerobic reaction. Some of our data have already been presented (31).

## Methods and Experimental

### *Chemicals*

Most of the amines used were purchased from Eastman Kodak Co., Fisher Scientific Co., and Brickman and Co. Other sources were as follows:

[1]*Manuscript received May 29, 1956.*
*Contribution from the Allan Memorial Institute of Psychiatry, McGill University, Montreal, Que. This work was aided by grants to Dr. R. A. Cleghorn from the Dominion–Provincial Mental Health Research Fund, and from the Foundations' Fund for Psychiatric Research, Yale University.*

adrenaline and noradrenaline from Winthrop-Stearns, Inc.; TTC[2] from Montclair Research Corp.; NTC from Nutritional Biochemicals Corp.; γ-aminobutyric acid, thioctic acid, and FMN from California Foundation for Biochemical Research; ATP, DPN, and FAD from Sigma Chemical Co.; GSH and GSSG from Schwartz Laboratories; and coenzyme A from Armour Research Division. The authors are grateful for the following donated materials: 5-hydroxytryptamine (serotonin creatinine sulphate) from Abbott Laboratories Ltd. and the Upjohn Co.; IIH from Hoffman-LaRoche Ltd.; 3-hydroxytyramine ("dopamine") from Dr. Karl Pfister III; atebrine from Dr. Lowell Hokin; 1,3,5-triphenylformazan and pyridoxal phosphate from Dr. W. W. Umbreit.

*Reaction Mixtures*

Several formulations were used in this work. The following reaction mixture was selected as the most convenient one: amine, *M*/10 (later reduced to *M*/50), 0.3 ml.; phosphate buffer, pH 7.4, *M*/5, 0.3 ml.; TTC, 2 mgm. or NTC[3], 1 mgm.; tissue suspension (Waring blendor) or extract (one part of tissue blended with 4 vol. water), up to 1.0 ml.; and water to a final volume of 2.0 ml. Occasionally the tissue was blended with *M*/100 phosphate buffer, pH 7.4, instead of with water. The ingredients were pipetted into 13 × 100 mm. test tubes, immersed in a well-stirred water-bath held at 37°. In control mixtures water was substituted for the amine solution; the formazan produced in these tubes represents endogenous reduction.

At the end of the incubation period 4 ml. *n*-butanol was added to each tube. The contents were shaken, spun in the centrifuge to separate the two phases (with the protein deposited at the interface), and the butanol was removed to a suitable cuvette for determination of the optical density. Acetone was occasionally used in the initial work, but it was found that it does not extract the formazan from the protein precipitate as well as butanol does. 1,3,5-Triphenylformazan was available for the establishment of a calibration curve, which proved to be linear up to at least 40 μgm./ml. at 485 mμ, using either the Beckman DU Spectrophotometer or the Coleman Junior Spectrophotometer. Results of experiments with TTC are thus reported in terms of the amount of reduced product. Those with NTC are given as optical density based on reading at 520 mμ.

The rate of amine dehydrogenation was estimated as the difference between the amount of formazan produced in the presence and absence of the substrate during a given period of time. Experimental conditions were adjusted so

[2]*The following abbreviations are used in the text: TTC, triphenyltetrazolium chloride; NTC, neotetrazolium chloride; IIH, isonicotinyl isopropylhydrazide = "Marsilid" phosphate; ATP, adenosine triphosphate; DPN, diphosphopyridine nucleotide; GSH and GSSG, reduced and oxidized glutathione, respectively; FMN, riboflavin-5'-phosphate; FAD, flavin adenine dinucleotide; IsoAA, isoamylamine; O.D.; optical density.*

[3]*NTC, a ditetrazolium compound, has the advantage that its reduction (both chemical and enzymatic) is not catalyzed by light (17) as may occur with TTC (16). Furthermore, it has been found that under comparable conditions for enzymatic reduction in the test system used in this laboratory NTC yields higher O.D. measurements than TTC does. The results, however, are qualitatively identical. After the earlier experiments described in this paper had been performed with TTC, NTC was substituted for it.*

that this difference, the "extra formazan", usually ranged from 20 to 200% of the endogenous reduction. Using TTC with three different types of rat tissue preparation the endogenous rates of formazan appearance were as follows:

| | *n* | Mean μgm./100 mgm. dry wt. per hr. | Standard error |
|---|---|---|---|
| Liver suspensions | 24 | 1.52 | 0.27 |
| Washed, acetonized liver powder | 9 | 0.22 | 0.01 |
| Brain suspensions | 45 | 0.61 | 0.05 |

From these data it can be seen that the suspensions of liver were the most active, but had a large associated standard error. Acetone treatment of the liver resulted in a reduced but more consistent activity. Suspensions of rat brain displayed intermediate values; these could be reduced by aging the brain tissue in the frozen state before blending.

When tissue was omitted from the reaction mixtures or when boiled tissue was added there was no reduction of tetrazolium in the usual experimental periods.

*Measurement of Amine Oxidase Activity*

The methods for determining amine oxidase activity manometrically and spectrophotometrically have been previously described (32).

## Results

*Time Curve*

Reduction of the tetrazolium salt is preceded by a lag phase (Fig. 1) during which no formazan can be detected. This usually happens even in the presence of the amine substrate. It has not yet been possible to eliminate this lag by use of any of a number of expedients, such as exhaustive nitrogenation of the reaction mixtures in Warburg flasks, gassing of the mixtures in the test tubes ordinarily used, conducting the reaction in an evacuated desiccator, or addition of ATP, DPN, or GSH. At the end of this lag period, the duration of which varies markedly from preparation to preparation, reduction is proportional to time of incubation up to a maximum time when the curves describing formazan production in the presence and absence of substrate reach a plateau.

*Substrate Concentration*

The rate of reaction increases with increasing substrate concentration to a maximum, beyond which higher concentrations of substrate inhibit the reaction (Fig. 2). These observations are in accord with those reported by Cotzias and Dole for amine oxidase, using tyramine or tryptamine (6, 7). Cotzias offers the hypothesis that the high substrate concentration contributes to the heat denaturation of the enzyme since the effect is not seen at 20° (6).

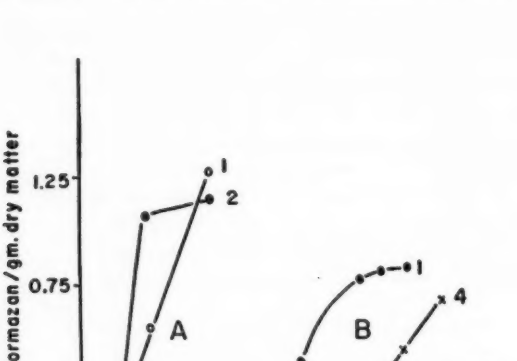


FIG. 1. Reduction of TTC by the amine dehydrogenating system as a function of time. Reduction mixtures as described in the text. Preparations (dry weight of tissue per tube in parentheses): A, curves 1 and 2, rat liver suspension (40 mgm.); curve 3, rat liver acetone powder (167 mgm.); tyramine used as substrate. B, curve 1, rat brain suspension (50 mgm.); curve 2, same (75 mgm.); curve 3, particulate matter of rat brain suspension sedimenting at 18,000 *g* in 20 min. (90 mgm.); curve 4, rat brain suspension, dialyzed three hours against water (40 mgm.); curve 5, same, but dialyzed 23 hr. (31 mgm.); isoamylamine used as substrate.

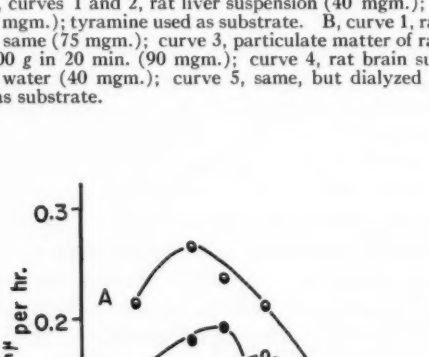


FIG. 2. Effect of substrate concentration on rate of NTC reduction. A, tryptamine; B, isoamylamine; C, tyramine.

*Tissue Concentration*

The reduction of tetrazolium salts proceeds very slowly when relatively small amounts of tissue are used. Appropriate further additions of tissue beyond 15 mgm. dry weight per tube bring about a sharp increase in formazan production per unit dry weight of tissue (Fig. 3), particularly with brain preparations. The occurrence of these two phases in the tissue concentration curves suggests that at least two factors, one of them present in limiting concentration, are necessary for the enzymatic reduction of tetrazolium salts by amines. From the curves shown in Fig. 3 it appears that a sufficient amount of this factor is present in 15–20 mgm. dry weight of brain or liver extract per tube to relieve the limitation on the velocity of the reaction. The possibility that two factors, at the least, are implicated in the dehydrogenation of amines led to further experiments described below (see *Washed Preparations*).

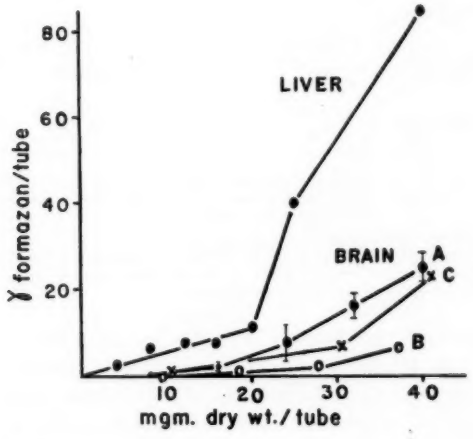


FIG. 3. Effect of tissue concentration on rate of TTC reduction. Brain: Curve A, each point is the mean of three or four determinations, vertical bars represent ± S. E.; curve B, washed residue of rat brain (see text); curve C, same but with boiled juice prepared from desiccated (Viobin) pig liver powder added in constant proportion to amount of brain tissue.

*Substrates*

It is seen in Table I that a variety of monoamines as well as some diamines can act as substrates in the reduction of tetrazolium. The diamines, however, were found not to act as substrates for washed tissue residues reconstituted with a liver extract (Series 4).

*Inhibition Studies*

Dehydrogenation of the amines in the system described presumably results in the formation of the corresponding aldehyde just as in the oxidative reaction, and this aldehyde should also be able to serve as a tetrazolium reductant. An attempt was made to eliminate such a reaction, should it occur and thereby

TABLE I

SUBSTRATES FOR AMINE DEHYDROGENATING SYSTEM OF BRAIN AND LIVER*

| | Series (number of determinations in parentheses) | | | |
|---|---|---|---|---|
| | 1(5) | 2(3) | 3(1) | 4(2) |
| | S. E. of the mean† | | | |
| Compound | .05 | .08 | | |
| β-Phenethylamine | 0.34 | | | |
| Tyramine | 0.28 | | 2.06 | |
| Dopamine | 0.70 | | | |
| Noradrenaline | 0.52 | | | |
| Adrenaline | 0.97 | | 1.70 | |
| Tryptamine | | | | 0.74 |
| 5-Hydroxytryptamine | | | | 0.87 |
| Isoamylamine | | 0.18 | 2.19 | 0.39 |
| 1,4-Butanediamine | | 0.31 | 0.49 | —‡ |
| 1,6-Hexanediamine | | 0.50 | 1.10 | —‡ |
| L-Lysine | | 0.54 | | —‡ |
| γ-Aminobutyric acid | | 0.36 | | —‡ |

NOTE.—*Series 1 and 2: rat brain suspensions; series 3: 20% rat liver suspension dialyzed against water at 2° for 15 hr.; series 4: rat brain suspension, thrice-washed residue, 32 mgm., plus boiled extract of 20 mgm. Viobin pig liver powder.*

**Data are given in mgm. extra formazan/gm. dry wt./hr. TTC was used in all series.*

†*The results were analyzed statistically by the method of analysis of variance (29). The S.E. shown is calculated from the data for any mean of the indicated number of determinations.*

‡*Endogenous reduction was inhibited by this substrate.*

contribute to formazan production, by adding a suitable carbonyl reagent. With cyanide (Table II) there was almost complete inhibition of the reaction, indeed much more than can be attributed to inhibition simply of the second reaction in a metabolic sequence. This is in contradiction to Francis's result with amine oxidase of liver demonstrated histochemically with NTC (10). Similarly, with a frozen liver suspension (Table II, Expt. 4) semicarbazide inhibited the amine dehydrogenation by about 80%. These results indicate the sensitivity of the amine dehydrogenating system of liver to cyanide and semicarbazide. On the other hand, in three experiments (not listed in the table) using IsoAA with frozen brain preparations, $M/100$ semicarbazide inhibited extra formazan production by only 5, 0, and 17%, respectively.

Hydrazine and hydroxylamine were themselves capable of stimulating greatly the reduction of NTC in the presence of liver extracts, an observation reminiscent of the finding of Colter and Quastel (5) that hemoglobin catalyzes oxidation–reduction reactions of hydroxylamine to form nitrogen, ammonia, and water. Further increases in the rate of reduction of the dye could be registered by adding IsoAA to the carbonyl reagent-containing mixtures, but no further investigations were made with these compounds.[4]

[4]*Solutions of hydrazine and hydroxylamine at pH's greater than physiological cause reduction of the tetrazolium salt (20).*

Atebrine was tested as an inhibitor because of the finding by Allegretti and Vukadinovic (1) that it inhibits monoamine oxidase action on adrenaline. It inhibited in the present system also (Table II, Expt. 3) with either IsoAA or tyramine as substrate.

Marsilid (IIH), a monoamine oxidase inhibitor (35), also affects brain preparations in the anaerobic reaction whether it is added *in vitro* to the reaction mixtures (Table II, Expt. 5) or injected into the rat some hours before the brain is excised at sacrifice (Expts. 6 and 7).

TABLE II

INHIBITION OF AMINE DEHYDROGENATING ACTIVITY OF RAT TISSUE SUSPENSIONS

| Expt. No. | Tissue | Substrate | Inhibitor | Final conc., moles/l. | Extra formazan* | Per cent inhibition |
|---|---|---|---|---|---|---|
| 1*a* | Liver | Tyramine | — | | 31.8 μgm. | |
| | | | Cyanide | $2 \times 10^{-2}$ | 0.3 | 99 |
| 1*b* | Liver | 1,6-Hexanediamine | — | | 10.4 μgm. | |
| | | | Cyanide | $2 \times 10^{-2}$ | 0.3 | 97 |
| 2 | Liver | IsoAA | — | | 39.4 μgm. | |
| | | | Cyanide | $10^{-5}$ | 46.1 | −17† |
| | | | | $10^{-4}$ | 33.4 | 15 |
| | | | | $10^{-3}$ | 7.6 | 81 |
| | | | | $2 \times 10^{-2}$ | 2.0 | 95 |
| 3*a* | Liver | IsoAA | — | | 37.8 μgm. | |
| | | | Atebrine | $10^{-3}$ | 11.2 | 71 |
| | | | " | $10^{-2}$ | 13.8 | 63 |
| 3*b* | Liver | Tyramine | — | | 35.3 | |
| | | | Atebrine | $10^{-3}$ | 18.6 | 47 |
| | | | " | $10^{-2}$ | 17.3 | 51 |
| 4 | Liver | IsoAA | — | | 0.548 O.D. | |
| | | | Semicarbazide | $7 \times 10^{-3}$ | 0.132 | 76 |
| | | | " | $2 \times 10^{-2}$ | 0.104 | 81 |
| 5 | Brain | IsoAA | — | | 0.057 O.D. | |
| | | | IIH | $10^{-4}$ | 0.041 | 28 |
| | | | " | $10^{-3}$ | 0.025 | 56 |
| 6 | Brain | IsoAA | — | | 0.097†O.D. | |
| | | | IIH | *in vivo* | 0.017§ | 82 |
| 7 | Brain | IsoAA | — | | 0.255 O.D. | |
| | | | IIH | *in vivo* | 0.065‖ | 74 |

NOTE.—*Expts. 1 and 2: dialyzed liver suspension, 120 mgm. wet wt. per tube, 35 min. incubation (means of 2 det. given); Expt. 3: dialyzed liver suspension, 100 mgm., 45 min.; Expt. 4: liver frozen and thawed before homogenizing, 120 mgm., 11 min.; Expt. 5: washed residue of homogenized frozen brain, 100 mgm., 30 min. (means of 5 det.); Expt. 6: brain suspensions prepared from 3 rats injected intraperitoneally with IIH, 6 μM. (= 13.8 mgm.)/100 gm. body wt., controls injected with saline at same time, body wt. = 140 ± 3 gm., sacrificed at 3 hr., NTC reducing activity based on 140 mgm. wet wt. of tissue, 30 min. incubation; Expt. 7: as in Expt. 6, except only one control rat; three receiving IIH, sacrificed at 20 hr. after injection.*

**In excess of endogenous production. TTC was used in Expts. 1–3; NTC in Expts. 4–7. (See Methods.)*

*†Stimulation observed with this concentration of cyanide.*

*‡Mean of 0.089, 0.117, 0.085.*

*§Mean of 0.015, 0.037, 0.000.*

*‖Mean of 0.048, 0.017, 0.130.*

*Concentration of Tetrazolium Salt*

Excessive amounts of NTC inhibit the dehydrogenation of IsoAA. The optimal concentration, other factors being held constant, is about 0.4 mgm./ml. in the reaction mixture. This is equivalent to $1.2 \times 10^{-3}$ *M*, expressed as the concentration of a *mono*tetrazolium group.

A similar phenomenon is seen when this tetrazolium salt is incubated with amine oxidase preparations. The enzyme is inhibited by NTC as shown by the rate of oxygen uptake or tryptamine disappearance (Table III). The reverse situation is also experimentally verified, viz., inhibition of tetrazolium reduction by oxygen. Precautions as mentioned above must be taken in order to exclude excess air from the reaction mixtures.

Inhibition of the tetrazolium reduction was not observed with TTC, even with concentrations as high as 1.3 mgm./ml. ($= 3.9 \times 10^{-3}$ *M*).

TABLE III

INHIBITION OF AMINE OXIDASE BY NEOTETRAZOLIUM CHLORIDE

| Expt. No. | Tissue | Substrate | Additions | Result |
|---|---|---|---|---|
| 1 | Frozen brain, once-washed residue | IsoAA | NTC, 0.6 mgm. | 0.120 extra O.D./70 min. |
| | | | 1.2 | 0.117 |
| | | | 1.8 | 0.019 |
| 2 | Fresh rat brain suspension (200 mgm.) | Tryptamine | — | 54 μl. $O_2$/60 min. |
| | | | NTC, 0.5 mgm. | 42 |
| | | | — | 4.6 μM./60 min.* |
| | | | NTC, 0.5 mgm. | 2.0 μM. |
| | | Tyramine | — | 125 μl. $O_2$/60 min. |
| | | | NTC, 0.5 mgm. | 47 |
| 3 | Rat brain, once-washed residue† (equivalent to 160 mgm. fresh tissue) | Tyramine | — | 73 μl. $O_2$/30 min. |
| | | | NTC, 0.3 mgm. | 34 |
| | | | 0.6 | 23 |
| | | | 0.9 | 34 |
| 4 | Rat brain suspension (140 mgm. tissue) | Tyramine | — | 70 μl. $O_2$/60 min. |
| | | | NTC, 1.0 mgm. | 35 |

**Method of substrate disappearance (32).*
†*This preparation of amine oxidase was inactive towards NTC.*

*Washed Preparations*

The rate of endogenous reduction of tetrazolium salts, by crude tissue suspensions, especially those of liver, was frequently so high that addition of an amine resulted in no or very little extra reduction. That is, the tissue minced in water with a Waring blendor (see *Methods*) already contained a high concentration of substrates which apparently saturate the pathways for tetrazolium reduction. However, it was found that about half the endogenous reduction could be eliminated if the particulate matter obtained by centrifuging such suspensions for 20 min. at 18,000 *g* and 2° was washed twice with water. At the same time the "extra formazan" produced was also reduced

somewhat but the resulting rates were nevertheless convenient for quantitative comparisons. The tissue supernatant fraction was incapable of reducing tetrazolium with or without added amine even in protracted periods of up to several hours.

The particulate fraction of rat brain suspensions exhibited erratic amine dehydrogenating activity, which was considerably reduced by three washings of the particles using the same conditions as for liver ("washed residue", Table IV). This result is quite different from that seen in manometric oxidation experiments where washed residues and dialyzed preparations retain the original amine oxidase activity (18, 30).

Attempts to regain the activity of washed brain residues led to the finding that the supernatant fraction, which had usually been discarded, contains a factor necessary for the amine dehydrogenating activity (Table IV, Expt. 1). Such a factor was found also in other tissues than brain, e.g. pig liver (Expts. 2 and 3) and rat liver (Expts. 4 and 5). A boiled aqueous extract of baker's yeast exhibited similar activity. A lyophilized powder prepared from rat liver boiled juice proved to be a potent and convenient source (Expt. 5).

Some characterization of the material possessing cofactor activity has been achieved. Thus, the activity in the various sources indicated above is retained after heating (Table IV). The active material passes through a cellophane membrane as demonstrated by the decreased activity of rat brain on prolonged dialysis (Fig. 1, cf. curves B4 and B5). Similarly, the cofactor activity of pig liver declines when the Viobin powder is dialyzed against water (Table IV, Expts. 2*e* and 2*f*). Activity is then found in the dialyzate used after lyophilization (Table IV, Expt. 2*g*). Activity of the pig liver factor is lost by ashing procedures (Table IV, Expts. 2*h* and 2*i*).

In attempting to characterize this cofactor, the following additives were tested for ability to catalyze the rate of tetrazolium reduction by amines, using washed particles of rat brain: ATP, DPN, FMN, FAD, pyridoxal phosphate, coenzyme A, GSH, GSSG, thioctic acid, cobaltous sulphate, cupric sulphate. In amounts of 0.5 and 1 mgm. per tube, none of these was effective.

## Discussion

The existence of a tetrazolium reducing system in which amines play the role of substrate at once raises the question of the relationship of this system to amine oxidase. The first possibility is that the components of the two systems are identical. This is supported by the location of both systems in the particulate matter of the tissues, by the similarity of the substrate concentration curves, and by the fact that both are inhibited by IIH and by atebrine. However, actual identity is excluded by the results with other inhibitors and by a cofactor requirement of washed brain and liver residues in the anaerobic, but not thus far demonstrated in the oxidative, reaction.

It is not unlikely that the oxidase and the anaerobic system are multiple in character. For example, they may have one enzyme in common but otherwise

TABLE IV

ACTIVATING EFFECT OF SUPPLEMENTS ON THE REDUCTION OF NTC IN PRESENCE OF RAT BRAIN PREPARATIONS AND ISOAMYLAMINE

| Expt. | Additions | Incubation time (min.) | Activity O.D./hr./100 mgm. dry wt. |
|---|---|---|---|
| 1*a* | Brain suspension, 40 mgm. | 35 | 0.390 |
| *b* | Brain, washed residue,* 32 mgm. | | 0.094 |
| *c* | Supernatant fraction of 1*a*, obtained by high speed centrifugation | | 0.000 |
| *d* | Residue + supernatant | | 0.378 |
| *e* | 1*c*, heated 10 min. at 100° | | 0.000 |
| *f* | Residue + 1*e* | | 0.498 |
| *g* | Heat-inactivated residue + 1*c* | | 0.000 |
| 2*a* | Brain suspension, 19 mgm. | 54 | 0.129 |
| *b* | Brain, washed residue, 28 mgm. | | 0.000 |
| *c* | Pig liver boiled juice, prepared from 10 mgm. Viobin powder | | 0.000 |
| *d* | Residue + 2*c* | | 0.184 |
| *e* | Residue + boiled juice prepared from suspension of Viobin powder which had been dialyzed 6 hr. against $H_2O$ | | 0.176 |
| *f* | Same as 2*e*, but dialysis prolonged to 24 hr. | | 0.088 |
| *g* | Residue + 8 mgm. of lyophilized dialyzate prepared from 8 gm. of Viobin powder, dialyzed 3 hr. against $H_2O$ | | 1.200 |
| *h* | Viobin powder, dry-ashed† ± residue | | 0.000 |
| *i* | Viobin powder, wet-ashed‡ ± residue | | 0.000 |
| 3*a* | Brain, washed residue, 30 mgm. | 75 | 0.026 |
| *b* | Pig liver boiled juice, prepared from 20 mgm. Viobin desiccated powder in 0.4 ml. | | 0.000 |
| *c* | Residue + 0.1 ml. juice | | 0.000 |
| *d* | " + 0.2 " " | | 0.067 |
| *e* | " + 0.3 " " | | 0.230 |
| *f* | " + 0.4 " " | | 0.463 |
| 4 | Brain, washed residue | 45 | 0.039 |
| | Same + boiled juice of rat liver obtained from 400 mgm. of fresh tissue | | 0.680 |
| 5*a* | Brain, washed residue, 28 mgm. | 45 | 0.095 |
| *b* | Lyophilized powder prepared from rat liver boiled juice, 50 mgm. | | 0.000 |
| *c* | Residue + 10 mgm. lyophilized powder | | 0.405 |
| *d* | " + 20 mgm. " " | | 0.600 |
| *e* | " + 30 mgm. " " | | 1.285 |
| *f* | " + 50 mgm. " " | | 2.000 |

**Residues in these experiments were thrice-washed with distilled water.*

†*The powder was reduced to ash in a muffle furnace; ash corresponding to 500 mgm. Viobin pig liver powder was used.*

‡*The boiled juice of Viobin powder was digested with 30% $H_2O_2$ over a flame until colorless, and then heating was continued until the material was negative for peroxide using erythrocyte catalase. The amount of material used per tube corresponded to 60 mgm. Viobin powder.*

differ in the pathway of transfer of electrons. The present experiments do not permit a decision as to the nature of the electron transport mechanism, but its terminus in the tetrazolium salts suggests the mediation of a flavoprotein, a number of which have been shown to catalyze the tetrazolium reduction. Brodie and Gots list some of these (4, see also 9); Green and his colleagues have used the dye in studying the metalloflavoprotein-catalyzed fatty acid dehydrogenations (12). Inhibition by atebrine further implicates a flavin since this drug exerts a specific action on flavoproteins (13, 27, 33).

Hitherto no prosthetic group has been resolved from amine oxidase, but the view that the enzyme possesses a flavin nucleotide in its structure has been proposed on several occasions. Indeed, the parallel with the amino acid oxidases, which are known to be yellow enzymes, is challenging: both of these enzymes catalyze the conversion of $=CH—NH_2$ to $=C=NH$ (or the corresponding cations formed by addition of a proton); both are efficiently carried out only under high partial pressures of oxygen, a characteristic of flavin enzymes; and both result in the formation of $H_2O_2$. Richter (22) in 1937 drew attention to some of these similarities in reaction mechanism. Hawkins (14) attempted to detect flavin involvement by comparing the activities of these two enzymes in the livers of riboflavin-deficient and normal rats; these experiments showed that the vitamin deficiency adversely affects the amine oxidase system. Govier *et al.* (11) postulated that the amine oxidase system includes the cytochromes, again implying the occurrence of a flavoprotein. Recently Mahler (19) has tentatively included amine oxidases in the group of yellow enzymes undergoing reoxidation directly by oxygen. The search for a flavin prosthetic group in amine oxidase has been stimulated further by evidence that the analogous enzyme, histaminase (diamine oxidase) contains FAD (reviewed in Ref. 34).

The requirement of a heat-stable, dialyzable factor, although thus far demonstrated only in the anaerobic reaction, indicates the complexity of the amine oxidase system, hitherto suspected but not directly demonstrated. This cofactor may be associated with a second protein, i.e. other than "amine oxidase", in the washed tissue residue. It has not been possible yet to identify it with any of a series of coenzymes and other catalysts tested. The inactivity of FMN and FAD is to be noted since this result appears to rule amine oxidase out of the family of flavoproteins. However, it is possible that riboflavin exists in coenzymatic forms other than the well-recognized mono- and di-nucleotides (cf. 15). Studies to elucidate the chemical nature and function of this cofactor are in progress.

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# SIZE, STABILITY, AND HETEROGENEITY OF APURINIC ACID[1]

By G. C. Wood[2] and David B. Smith

## Abstract

Apurinic acid prepared by mild acid treatment of sodium desoxyribonucleate and of fractions of sodium desoxyribonucleate was sufficiently stable to permit estimations of molecular weight and polydispersity. Apurinic acid from unfractionated desoxyribonucleate had a weight-average molecular weight of 25,000 and was very polydisperse. Preparations from fractionated desoxyribonucleate representing about half the original nucleic acid were much less polydisperse and had molecular weights of about 10,000.

## Introduction

Investigation of the structure of partially degraded desoxyribose nucleic acid (DNA) is a potential means of elucidating the structure of DNA itself and work employing degradation by desoxyribonuclease, dilute alkali, dilute acid, and heat has been summarized recently (7). In the present paper attention is restricted to substances prepared by acid treatment of calf-thymus DNA.

Acid hydrolysis of DNA yields ultimately phosphate, desoxyribose, purines, and pyrimidines. Mild acid treatment, however, produces a variety of intermediate products depending on the severity of the treatment. When a DNA solution is acidified through a dialysis membrane to pH 2.6 the molecules contract reversibly (16). If the acid is added dropwise the physical properties of the solution change irreversibly (6, 9), whilst treatment at pH 1.7 for 24 hr. at 37° C. removed purines from the polymer molecule. The product of this reaction, apurinic acid (APA), has been studied extensively by Tamm *et al.* (20, 21, 22, 23). Under their conditions the purines were removed from DNA quantitatively; no dialyzable nucleotides were produced and only a small amount of dialyzable phosphorus was formed (21). Lucy and Kent (14), on the other hand, found that the purines were not removed completely under similar conditions and also detected free thymine and cytosine in the reaction mixture. Laland (13) has reported that the purines are removed completely when a solution of DNA is treated with a cation exchange resin in the hydrogen form.

Tamm and Chargaff (20) state that the weight-average molecular weight of a sample of APA was 15,000 from light-scattering measurements* and

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**This value was calculated using a value of 0.160 ml./gm. for the specific refractive increment, a value earlier reported for DNA (24). Using the more recently determined value for the specific refractive increment of DNA, viz. 0.188 ml./gm. (17), the molecular weight is reduced to 13,000—M. E. Reichmann, private communication.*

15,500 from sedimentation and diffusion measurements. The material showed only a single electrophoretic component over the limited range of conditions of their experiments.

A study of APA prepared by two methods from each of two samples of DNA and from fractionated DNA was undertaken to get more information on its stability, size, and heterogeneity.

## Materials and Methods

*Preparation of DNA*

Two batches of DNA (DNA IV and DNA V) were prepared from fresh calf-thymus gland by the method of Smillie, Marko, and Butler (18) with the following modifications:

(1) In both preparations the nucleoprotein was extracted at +4° C. instead of −10° C.

(2) DNA IV was precipitated from a nucleoprotein solution whose concentration was four times that recommended by Smillie *et al.* The crude DNA IV still contained protein and to remove this the precipitate was dissolved in water and the sodium chloride concentration adjusted at 0.14 *M*. This caused precipitation of nucleoprotein, which was removed by high speed centrifugation. DNA IV was then precipitated with alcohol after the salt concentration was adjusted to 3 *M*. The yield of DNA IV based on the original weight of wet gland was 65% of that of DNA V.

The nitrogen/phosphorus ratio (w/w) was the same for both samples (1.65 ± 0.03) but the ratios of the extinction coefficients at 230 mμ and 260 mμ differed slightly (2.35 for DNA IV, 2.09 for DNA V). As described later the two preparations were found to differ also in other respects.

*Preparation of APA*

(*a*) *Method I, Based on Tamm's Procedure* (*21*)

One volume of 0.1 *N* hydrochloric acid, added slowly with stirring to five volumes of 0.25% DNA in 0.05 *M* potassium chloride, brought the pH to 1.7 and produced a heavy precipitate. After storage at 37° C. for 16 hr. with occasional stirring, most of the precipitate had dissolved. Following dialysis against 0.2 *M* borate buffer, pH 7.3 at 4° C., and then several changes of water, a small residue was centrifuged down and discarded and the supernatant was freeze-dried.

(*b*) *Method II, Based on Laland's Procedure* (*13*)

Duplicate samples of 0.1% DNA solution were treated with Amberlite 1R-120 ($H^+$), one at 36° C. and the other at 25° C. At intervals, 3 ml. aliquots of each were diluted and adjusted to pH 5.0 for optical density measurements at 260 mμ. Since purines are absorbed to the resin as they are split off, these measurements (Fig. 1) show the course of the reaction. The curves extrapolate at zero time to a point 30% higher than the absorption

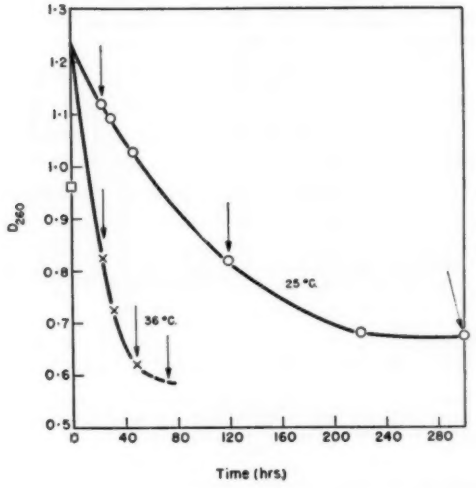


FIG. 1. Change of ultraviolet absorption ($\lambda = 260$ m$\mu$) during treatment of DNA with Amberlite IR-120 ($H^+$). The point, □, at zero time shows the absorption of the untreated DNA solution.

of untreated DNA. This increase is consistent with other values reported for the hyperchromic effect occurring when the structure of DNA is altered by physical and chemical agents (10).

At the times indicated by arrows, 40 ml. portions were withdrawn, neutralized, centrifuged, and freeze-dried.

*Fractionation of DNA*

The following procedures, based on the work of Mathieson and Porter (15), were used several times and the fractionation patterns were found to be reproducible.

Hydrochloric acid (0.2 *N*) was added dropwise in stages to 0.2% DNA solutions in water at 4° C. After each addition, any precipitate was separated by centrifugation, washed with ethanol, and ether- and air-dried. From DNA IV, fractions A1 to A6, prepared as represented in Fig. 2*a*, comprised 71% of the starting material.

This procedure precipitated only 12.5% of DNA V (fractions B1 to B4) (Fig. 2*b*). More DNA was precipitated by the subsequent addition of ethanol (fractions B5 to B7) (Fig. 2*b*).

The addition of 0.2 *N* hydrochloric acid containing 1.0 *M* sodium chloride to DNA V dissolved in 0.2 *M* sodium chloride gave fractions C1 to C3 (Fig. 2*c*) comprising 41% of the starting material.

Apurinic acid was prepared from these fractions by Method I.

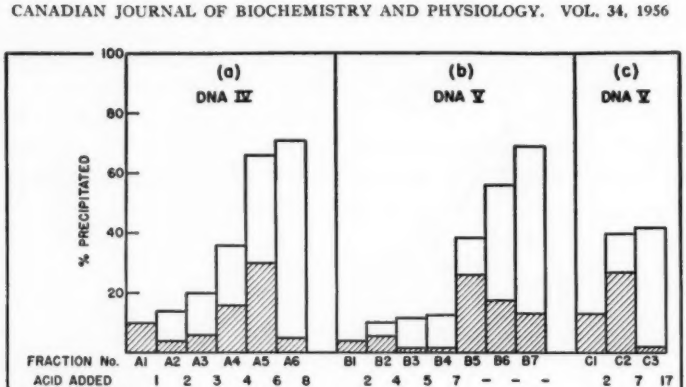
(a)
DNA IV
(b)
DNA V
(c)
DNA V
% PRECIPITATED
100
80
60
40
20
FRACTION No. A1 A2 A3 A4 A5 A6 B1 B2 B3 B4 B5 B6 B7 C1 C2 C3
ACID ADDED (mls./50mls. soln.) 1 2 3 4 6 8 2 4 5 7 - - - 2 7 17
ETHANOL ADDED (mls./50mls. soln.) - - - - - - - - - - 5 15 35 - - -

FIG. 2. Fractionation of DNA. The shaded areas are proportional to the weight of DNA precipitated in each step. The sums of shaded and unshaded areas are proportional to the total weight precipitated at the end of each step.

*Physical Measurements*

A sodium acetate buffer, pH 5.0, $\mu = 0.15$, was used. The specific refractive increment ($dn/dc$) and partial specific volume ($\bar{V}$) were assumed to be the same as for DNA, viz. 0.188 ml./gm. (17) and 0.55 ml./gm. (5) respectively.

(*a*) *Ultracentrifugation*

Archibald's treatment (2) of transient ultracentrifuge data was applied as described elsewhere (19). According to this treatment at values of $r$ corresponding to the locations of the meniscus of the solution and the base of the cell respectively,

$$(1/rc)(dc/dr) = \delta_m(\text{or } \delta_b) = \{M_m(\text{or } M_b)\,(1 - \bar{V}\rho)\omega^2\}/RT$$

where $r$ is the distance from center of rotation, $c$ the corresponding concentration, $M$ the weight-average molecular weight, $\rho$ the density of the solvent, $\omega$ the angular velocity of the rotor, $R$ the gas constant, and $T$ the absolute temperature. The subscripts $m$ and $b$ refer to conditions at the meniscus and base respectively.

For a monodisperse material whose sedimentation and diffusion rates are not concentration dependent, $\delta_b$ and $\delta_m$ are equal. Polydispersity leads to values of $\delta_b$ higher than $\delta_m$ and the weight-average molecular weight ($M_w$) may be obtained by extrapolation to zero sedimentation period (2). Concentration dependence usually causes a greater depression of $\delta_b$ than of $\delta_m$ but this effect may be eliminated by extrapolation to zero concentration (19). The ratio $\delta_b/\delta_m$ may be used empirically for comparing polydispersities where centrifuging conditions, times, and concentrations are similar.

The Spinco Model E ultracentrifuge using the standard 12 mm. cell at 12,590 r.p.m. was used in the Archibald treatment. The rotor temperature

varied during the run by about 2° C. and the average value was used. For sedimentation velocity experiments, the Spinco synthetic boundary cell was used at 59,780 r.p.m. Sedimentation coefficients were computed and corrected to water at 20° C. by the usual methods (1).

(*b*) *Light-scattering Measurements*

Solutions were cleaned by passage through an ultrafine sintered glass filter and the intensity of the light scattered at 90° was measured in the Brice-Phoenix light-scattering photometer. The cell, of the semicylindrical partition type, was calibrated relative to the standard square cell, using Ludox after the method of Doty and Bunce (11). Weight-average molecular weights were calculated using the usual equation,

$$Kc/R_{90} = 1/M + Bc\,,$$

in which $R_{90}$ is Rayleigh ratio at 90°, $c$ concentration (gm./ml.), $B$ second virial coefficient,

$$K = \frac{2\,\pi^2\,n^2\,(dn/dc)^2}{N\lambda^4},$$

where $n$ is refractive index of solvent, $dn/dc$ specific refractive increment, $N$ Avogadro's number, $\lambda$ wavelength of light (4370 Å).

*Chemical Analysis*

The phosphorus content of six samples of APA, estimated by the method of King (12) on material dried *in vacuo* over phosphorus pentoxide at 90° C., was 11.3 ± 0.2%. The concentration of solutions used for physical measurements was calculated from their phosphorus content.

The purine and pyrimidine contents relative to phosphorus content were determined by the method of Wyatt (25).

## Results and Discussion

*Stability of APA*

Since the Archibald ultracentrifuge experiments required about 18 hr., the stability of APA over this length of time was tested. The intensity of light scattered at 90° by a 0.245% solution of APA prepared from DNA V by Method I was measured as a function of time at room temperature. The results are given below:

| $t$ (hr.) | $Kc/R_{90}$ |
|---|---|
| 0.5 | $4.58 \times 10^{-5}$ |
| 17.0 | $4.76 \times 10^{-5}$ |
| 41.0 | $5.10 \times 10^{-5}$ |

A separate experiment showed that at 0.5 hr. the second virial coefficient was positive but very small and that it increased slightly with time. Thus the weight-average molecular weight falls by less than 3% in 17 hr.

*Molecular Weight of APA*

Preliminary ultracentrifuge experiments showed that APA is polydisperse and that its sedimentation rate (Fig. 4) depends on concentration. The weight-average molecular weight of a sample of APA prepared from DNA V by Method I was found (19) to be 27,000 ± 3000 by Archibald's method. Light-scattering measurements (Fig. 3) on this preparation gave a weight-average molecular weight of 25,000 ± 500. The ratio $\delta_{b_{c\to 0}}/\delta_{m_{c\to 0}}$ for this sample increased from 2.1 after 4.2 hours centrifugation at $\omega$ = 1318 rad./sec. to 3.1 after 17.0 hours. The value of $\delta_b$ was very concentration dependent (19). The value of $\delta_m$, however, showed little concentration dependence and approached a value corresponding to a molecular weight of 7000.

Other preparations of APA from DNA V and also from DNA IV had similar average molecular weights and showed similar polydispersity.

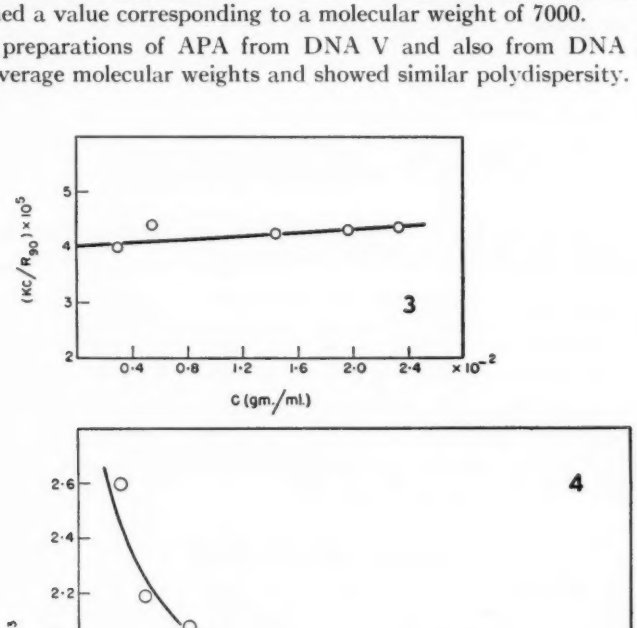


FIG. 3. Light scattering by APA prepared from DNA V by Method I. Solvent—sodium acetate buffer, pH 5.0, ionic strength 0.15.

FIG. 4. Sedimentation coefficients of APA prepared from unfractionated DNA V (○) and from the fractions B4 (□), B5 (△), and B6 (▽). Solvent—sodium acetate buffer, pH 5.0, ionic strength 0.15.

TABLE I

ULTRACENTRIFUGE DATA ON APA SAMPLES PREPARED AFTER DIFFERENT REACTION TIMES

| Time of reaction, hr. | Time of centrifuging, hr. | $c_m$, % | $c_b$, % | $\delta_m$ | $\delta_b$ |
|---|---|---|---|---|---|
| 16 | 5.3 | 0.28 | 2.09 | 0.94 | 1.17 |
| | 8.5 | 0.23 | 2.49 | 0.68 | 0.96 |
| | 16.5 | 0.12 | 2.86 | 0.55 | 0.72 |
| 40 | 4.3 | 0.45 | 0.90 | 0.35 | 0.47 |
| | 8.5 | 0.42 | 1.00 | 0.34 | 0.47 |
| | 17.0 | 0.35 | 1.20 | 0.29 | 0.47 |
| 64 | 4.3 | 0.49 | 1.28 | 0.45 | 0.60 |
| | 12.9 | 0.46 | 1.70 | 0.30 | 0.56 |
| | 20.0 | 0.32 | 2.00 | 0.34 | 0.49 |

Since part of the polydispersity of APA might arise from incomplete reaction of the DNA, the effect of increasing the reaction time was studied. Table I shows results of single ultracentrifuge runs on each of three samples of APA. The differences between $\delta_m$ and $\delta_b$ show that even after a reaction time of 64 hr., considerable polydispersity remains. Precise comparisons cannot be made since the concentration was not the same in these three runs and the concentration dependences of the materials were not determined.

Table II shows the results of single ultracentrifuge runs on APA prepared by Method II. The difference between $\delta_b$ and $\delta_m$ is greater than for APA prepared by Method I, indicating that the products of Method II are the more polydisperse. The extent of the polydispersity is so great that $\delta_b$ could not be estimated precisely because of the steepness of the ultracentrifuge patterns near the base. In the samples prepared at 25° C., however, the values of $\delta_m$ are similar to those found with samples prepared by Method I whilst preparation at 36° C. apparently leads to lower values. The values of $\delta_b$ in the preparation resulting from 300 hr. reaction at 25° C. are high because $c_b$ is considerably lower than in the other two runs.

The ultraviolet data in Fig. 1 indicate that the different preparations by Method II have different purine contents. Purines could not be detected by paper chromatography in acid hydrolyzates (25) of the products of 300 hr. treatment at 25° C. and 72 hr. at 36° C. Assuming that the two curves in Fig. 1 do not level off to the same value of $D_{260}$ because some pyrimidines are removed at 36° C., the fraction of the total purines of DNA which remain in each of the preparations may be calculated from the U.V. data; the results are included in Table II. Thus although after 24 hr. reaction at 25° C. 80% of the purines remain, the molecular weight had already fallen to less than 50,000. There thus seems to be little correlation between removal of purines and fall of molecular weight.

TABLE II

ULTRACENTRIFUGE AND ANALYTICAL DATA FOR APA PREPARED BY METHOD II

| Time of reaction, hr. | Time of centrifuging, hr. | $c_m$, % | $c_b$, % | $\delta_m$ | $\delta_b$ | Purines remaining, % |
|---|---|---|---|---|---|---|
| (*a*) *Reaction at 25° C.* | | | | | | |
| 24 | 6.5 | 0.40 | 1.3 | 0.53 | 1.8 | 80 |
| | 11.0 | 0.34 | 1.4 | 0.52 | 1.7 | |
| | 15.3 | 0.33 | 1.5 | 0.45 | 1.7 | |
| | 19.5 | 0.30 | 1.5 | 0.44 | 1.8 | |
| 120 | 3.8 | 0.44 | 0.94 | 0.36 | 0.60 | 27 |
| | 16.3 | 0.32 | 1.30 | 0.34 | 0.58 | |
| | 17.5 | 0.32 | 1.16 | 0.32 | 0.56 | |
| 300 | 4.0 | 0.12 | 0.37 | 0.36 | 1.7 | 0 |
| | 17.0 | 0.18 | 0.68 | 0.28 | 2.3 | |
| | 18.0 | 0.10 | 0.69 | 0.28 | 2.4 | |
| (*b*) *Reaction at 37° C.* | | | | | | |
| 24 | 6.0 | 0.44 | 0.80 | 0.21 | 0.85 | 29 |
| | 10.3 | 0.42 | 0.80 | 0.18 | 0.80 | |
| | 14.7 | 0.41 | 0.91 | 0.12 | 0.68 | |
| | 19.0 | 0.41 | 0.92 | 0.11 | 0.70 | |
| 48 | 3.5 | 0.28 | 0.66 | 0.53 | 0.75 | 0 |
| | 15.5 | 0.17 | 0.90 | 0.51 | 0.77 | |
| | 16.3 | 0.17 | 0.90 | 0.49 | 0.81 | |
| 72 | 7.0 | 0.47 | 0.96 | 0.25 | 0.52 | 0 |
| | 17.0 | 0.42 | 1.04 | 0.19 | 0.44 | |

*Apurinic Acid Prepared from Fractionated DNA*

The polydispersity of APA may be due to the heterogeneity of DNA. Samples of APA were, therefore, made from a series of DNA fractions.

The sedimentation coefficients of APA prepared from three fractions of DNA V are shown in Fig. 4. They are lower than those of APA prepared from unfractionated DNA (Fig. 4) and do not vary appreciably with concentration. Molecular weights could, therefore, be calculated by Archibald's method from ultracentrifuge measurements at a single concentration. The results are shown in Table III where the sample numbers correspond to the fraction numbers in Fig. 2.

The values of $\delta_b/\delta_m$ indicate that all the samples are much less polydisperse than APA from unfractionated DNA. The molecular weights of these materials are lower than the molecular weight of APA from unfractionated DNA; they represent, however, only about 50% of the total DNA.

Chemical analyses of samples of APA prepared from DNA fractions are shown in Table IV. Both B1 and B2 are only partially apurinated under conditions where the purines are almost completely removed from the other fractions. There is some indication that the various fractions differ in their thymine/cytosine ratios.

TABLE III

ULTRACENTRIFUGE DATA FOR APA SAMPLES PREPARED FROM DNA FRACTIONS

| Sample | Time of centrifuging, hr. | $c_b$, % | $c_m$, % | $\delta_b$ | $\delta_m$ | Mol. wt. | $\delta_b/\delta_m$ |
|---|---|---|---|---|---|---|---|
| A3 | 4.5 | 0.59 | 0.34 | 0.40 | 0.36 | 11,000 | 1.1 |
| | 8.5 | 0.73 | 0.31 | 0.42 | 0.32 | | 1.3 |
| | 13.0 | 0.67 | 0.26 | 0.42 | 0.36 | | 1.2 |
| | 17.2 | 0.70 | 0.24 | 0.44 | 0.38 | | 1.2 |
| A5 | 0.5 | 2.23 | 1.81 | 0.33 | 0.29 | 9,000 | 1.1 |
| | 4.3 | 2.62 | 1.56 | 0.32 | 0.26 | | 1.2 |
| | 9.0 | 2.85 | 1.43 | 0.30 | 0.27 | | 1.1 |
| | 13.2 | 3.00 | 1.20 | 0.31 | 0.30 | | 1.1 |
| | 17.5 | 3.07 | 1.24 | 0.30 | 0.25 | | 1.2 |
| B5 | 10.1 | 1.24 | 0.57 | 0.36 | 0.29 | 10,400 | 1.2 |
| | 14.3 | 1.38 | 0.56 | 0.38 | 0.25 | | 1.5 |
| | 18.4 | 1.39 | 0.57 | 0.32 | 0.18 | | 1.8 |
| B6 | 13.3 | 1.26 | 0.61 | 0.31 | 0.21 | 10,100 | 1.5 |
| | 17.4 | 1.29 | 0.60 | 0.29 | 0.18 | | 1.6 |
| | 21.9 | 1.30 | 0.60 | 0.26 | 0.13 | | 2.0 |
| C1 | 8.9 | 0.93 | 0.38 | 0.42 | 0.36 | 11,300 | 1.1 |
| | 13.1 | 0.93 | 0.41 | 0.42 | 0.36 | | 1.1 |
| | 16.2 | 1.01 | 0.33 | 0.43 | 0.38 | | 1.2 |
| C2 | 8.9 | 0.98 | 0.48 | 0.32 | 0.32 | 9,500 | 1.0 |
| | 13.1 | 1.06 | 0.43 | 0.32 | 0.33 | | 1.0 |
| | 16.2 | 1.10 | 0.39 | 0.33 | 0.33 | | 1.0 |

TABLE IV

ANALYSES OF APA SAMPLES PREPARED FROM DNA FRACTIONS

| Sample | $\frac{\text{(Thymine)}}{\text{(Cytosine)}}$ | $\frac{\text{(Thymine) + (cytosine)}}{\text{(Phosphorus)}}$ | $\frac{\text{(Adenine) + (guanine)}}{\text{(Phosphorus)}}$ |
|---|---|---|---|
| DNA V | 1.36 | 0.45 | 0.46 |
| | 1.34 | 0.46 | 0.47 |
| B1 | 1.36 | 0.65 | 0.26 |
| | 1.32 | — | — |
| B2 | 1.37 | 0.89 | 0.23 |
| | 1.37 | 0.68 | 0.18 |
| | 1.31 | — | — |
| | 1.29 | — | — |
| B4 | 1.23 | 0.44 | 0.09 |
| | 1.24 | 0.48 | 0.11 |
| B5 | 1.17 | 0.50 | 0.13 |
| | 1.18 | 0.48 | 0.13 |
| B6 | 1.18 | 0.54 | 0.13 |
| | — | 0.50 | 0.13 |
| B7 | 1.32 | 0.52 | 0.05 |
| | 1.30 | 0.49 | — |
| C1 | 1.36 | 0.53 | 0.05 |
| | 1.31 | 0.59 | 0.06 |
| C2 | 1.34 | 0.60 | 0.05 |
| | 1.30 | 0.41 | 0.04 |
| C3 | 1.46 | 0.56 | — |
| | 1.46 | — | — |

## Conclusions

Apurinic acid was found to be sufficiently stable for satisfactory physical measurements. The weight-average molecular weight of a sample of APA was 27,000 from ultracentrifuge data and 25,000 from light-scattering experiments; other samples had similar molecular weights. These values are a little higher than those reported by Tamm and Chargaff (20) and were not affected appreciably by prolonging the treatment of DNA with acid. The material was polydisperse and some of it was of molecular weight at least as low as 7000. Polydispersity of the starting material contributes to the polydispersity of APA. Samples of APA prepared from fractions of DNA, representing 40–50% of the total, had molecular weights ranging from 9000 to 11,300. The average size of APA molecules is smaller than that suggested by Dekker and Schachman (10) for polynucleotides.

APA is probably formed by the breaking of certain acid-labile bonds occurring at intervals along the polynucleotide chains. Prolonged reaction of DNA with acid does not appear to decrease the polydispersity of the product so that the polydispersity is probably not due to incomplete fission of these acid-labile bonds. APA prepared from DNA fractions are, however, much less polydisperse, indicating that in these fractions, representing about half the DNA, the labile bonds are more uniformly distributed. These observations suggest that DNA may consist of different types of polynucleotide chains in each of which acid-labile bonds are uniformly distributed but which differ in the frequency at which the acid-labile bonds occur. There is already ample evidence that DNA is chemically heterogeneous (3, 4, 8) and it is interesting to note that the samples of APA prepared from DNA fractions differ slightly in the ratio of their thymine and cytosine contents, implying that acid precipitation separates DNA into fractions of different composition.

These speculations could be further investigated by employing alternative methods of fractionating DNA whilst the development of methods for determining end groups would facilitate the identification of the acid-labile bonds.

## Acknowledgment

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# THE EFFECT OF AUREOMYCIN ON THE PRODUCTION OF HEMORRHAGIC SHOCK IN THE RAT[1]

By H. G. Downie

## Abstract

Hemorrhage into the jejunum and ileum, especially in those areas where there were ingesta, was observed in an earlier study designed to standardize a method for the production of hemorrhagic shock in the rat. This type of lesion might permit entry of bacteria and toxins, thus fatally increasing the strain on the animal. In the present study aureomycin was administered orally, 50 mgm. daily in a single dose for three days, and the rats were submitted to the standardized shock procedure. In a second series the same procedure was followed but a fourth dose of 50 mgm. of aureomycin was administered five hours before hemorrhage. None of the indices measured indicated that aureomycin used in such a manner was effective in reducing the mortality, or the hemorrhage into the bowel.

## Introduction

During earlier experiments designed by Downie and Stevenson (5) to standardize a method for the production of hemorrhagic shock in the rat, it was noticed that in many cases hemorrhage occurred in the bowel especially in those areas containing ingesta. The present series of experiments was designed to investigate whether aureomycin could reduce the intestinal damage when administered before shock.

Numerous reports have appeared on the effects of shock on the intestinal tract. Blalock (2) observed hemorrhage in the bowels of dogs after their blood pressure had been maintained at 70 mm. Hg for three hours by administration of acetylcholine. Selye (12) considered ulceration in the stomach and intestine a conspicuous feature of his alarm reaction and suggested that this would permit the entry of substances normally retained in the bowel by an intact epithelium. Others (10, 3) have also emphasized the gastrointestinal ulceration which may be produced by non-specific damage and shock. Hemorrhagic shock can produce marked pathological changes in jejunum and ileum of the dog (13) and copious amounts of bloody exudate have been observed in the stomach and small gut of the rat in this condition (11). The exudation is increased by transfusion of blood or plasma indicating an increased permeability (1). In man, acute peptic ulcer has been recognized as a possible complication of major surgery (7) and Davis, Wetzel, and Davis (4), who have encountered gastrointestinal hemorrhage and ulceration following neurosurgical operations, suggest that these represent an extreme physiological response to the physical stress of surgery wherever its location.

Fine, Frank, Schweinburg, Jacob, and Gordon (6, 8) found that pretreatment with aureomycin improved the outcome for dogs subjected to hemorrhagic shock. On the other hand, using a somewhat similar procedure, Williams and Clarke (14) and Hardy, Morris, Yow, Haynes, and DeBakey (9) could show no significant effect of aureomycin.

[1]*Manuscript received May 28, 1956.*
*Contribution from the Department of Physiology, Ontario Veterinary College, Guelph, and the Department of Physiology, University of Western Ontario, London, Canada.*

## Method

The method used was that of Downie and Stevenson (5). Male Sprague–Dawley rats weighing approximately 400 gm. were given nembutal (4 mgm. per 100 gm. body weight) intraperitoneally. The carotid artery was cannulated for blood pressure recording and heparin was administered by injection (2.5 mgm. in 0.1 ml. distilled water). The artery was then attached to a Sanborn electromanometer and "Polyviso" recorder. The jugular vein was cannulated and connected to an adjustable reservoir. Forty-five minutes after the administration of the nembutal, blood was withdrawn from the rat by lowering the reservoir and, over a period of 10 min., the blood pressure was reduced from approximately 160 mm. Hg to 30 mm. Hg. This "Initial Bleeding Volume" was recorded. By subsequent small hemorrhages, the blood pressure was maintained at 30 mm. Hg until there was (*a*) a reduction in the volume of blood in the reservoir at three successive two-minute intervals (automatic reinfusion), (*b*) respiratory failure followed by a sudden drop in blood pressure or, (*c*) excessive excursions in the mean blood pressure. At this time the reservoir blood was returned as a transfusion over the 10-min. period. Pressure recordings were maintained for a short period of time after reinfusion at the end of which time the vessels were tied off, and the animal was returned to its cage. A survival criterion of 48 hr. was used.

Aureomycin hydrochloride (Lederle) was given orally in a single daily dose to the experimental animals, otherwise the experimental and control animals were treated in the same way. Food and water were always available.

## Results

Of six rats given 50 mgm. aureomycin daily for three days and subjected to the standardized shock on the fourth day, five died and one survived. Six control animals showed a similar mortality.

To ensure that the dose of aureomycin was adequate, another series of six rats was given 50 mgm. aureomycin daily for three days and further 50 mgm. aureomycin five hours before hemorrhagic shock was induced on the fourth day. Again five died and one survived; in the control group four died and two survived.

Hemorrhage into the digestive tract occurred in all four groups regardless of treatment. Of the 24 animals studied such hemorrhage was seen in 15, with the following distribution by organs: stomach, 7; duodenum, 1; jejunum, 12; ileum, 2; and caecum, 2. Ulceration of the stomach was noted in 9 animals, and hemorrhage in the lungs of 9. In 19 animals, death occurred within 48 hr., and was generally preceded by convulsions. Aureomycin treatment had no effect on the incidence of these findings, or on any of the other parameters measured (arterial pressure, bleeding volume, duration of hypotension, heart rate, respiration, or rectal temperature).

Following death of an animal bacterial cultures, both aerobic and anaerobic (Brewer's anaerobic jar), were made from the intestinal tract and other

organs. The cultures were made on 5% sheep blood agar plates using standard bacteriological techniques. There was no significant reduction in the number of colonies obtained from the aureomycin-treated animals compared to the controls. There appeared, however, to be a shift toward Gram-positive colonies in the upper intestinal tract of the animals which had received aureomycin.

## Discussion and Conclusions

Gastrointestinal hemorrhage appears to be a critical complication of severe hemorrhagic shock in the rat. Its cause may be related to the mechanical erosion by the contents when the mucosa is in an hypoxic and debilitated state.

In the present study pretreatment with aureomycin did not reduce the mortality from irreversible hemorrhagic shock in the rat. This indicates that the fatal effects of this procedure in the rat are not likely due to invasion by, or intoxication from, aureomycin-sensitive organisms. Similarly the gastrointestinal hemorrhage and erosion seen in this condition cannot be attributed to such organisms. Although the dog may differ in its gastrointestinal flora, doubt has also been thrown on the therapeutic importance of antibiotics in hemorrhagic shock in this species (14, 9).

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# THE FORMATION OF 2-PYRROLECARBOXYLIC ACID FROM HYDROXY-D- AND ALLOHYDROXY-D-PROLINE[1]

By G. Letellier and L. P. Bouthillier

## Abstract

Evidence is presented for the identity of chemically prepared 2-pyrrolecarboxylic acid and the metabolite formed in rat and human tissues from hydroxy-D-proline and allohydroxy-D-proline. 2-Pyrrolecarboxylic acid is not an intermediate in the biological degradation of natural hydroxyproline.

## Introduction

A recent report by Radhakrishnan and Meister (4) that a purified sheep kidney D-amino acid oxidase preparation favors, in the presence of catalase, the oxidation of hydroxy-D-proline and allohydroxy-D-proline to 2-pyrrolecarboxylic acid has prompted the publication of the present paper. In the course of a study on the metabolism of hydroxy-DL-proline-2-$C^{14}$ in rat tissues, we observed the formation of a radioactive catabolite which we identified as 2-pyrrolecarboxylic acid (PCA). This product was characterized in rat kidney and liver homogenates which had been incubated in the presence of the labelled amino acid. It was also found in the urine of rats which had been injected intraperitoneally with hydroxy-DL-proline-2-$C^{14}$. The results indicate that PCA originates from hydroxy-D-proline but not from the natural isomer. Allohydroxy-D-proline is also a precursor of PCA. This catabolite was also detected in the urine of human subjects who had taken orally hydroxy-D-proline or allohydroxy-D-proline. A detailed description of these experiments is presented in this communication.

## Methods and Materials

*1. Incubation Techniques*

*(a) Tissue Homogenates*

About one gram of rat liver or kidney tissue was homogenized in a mixture of 3 ml. of 0.04 *M* disodium phosphate and 2 ml. of 0.04 *M* magnesium phosphate, adjusted to pH 7.5 with sodium hydroxide. To the homogenate was added 5 to 10 mgm. of hydroxyproline as substrate and the preparation was incubated for four hours at 37°. At the end of the incubation period, the enzymatic reaction was stopped by adding ethanol and heating 20 min. on a water bath. The proteins were separated by centrifugation and the supernatant was assayed for the presence of catabolites by the use of paper chromatography, specific coloring reagents, or radiochromatography.

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*(b) Tissue Slices*

The liver or the kidney of a rat was placed between two glass plates chilled over ice and thin slices were cut freehand with a razor blade. About 250 mgm. of slices were suspended in 5 ml. of Krebs–Ringer phosphate buffer (pH 7.4) containing 5 to 10 mgm. of hydroxyproline as substrate. Incubation was carried out in an atmosphere of air or oxygen at 37° for four hours. The tissue slices were pulverized in the incubation medium and trichloroacetic acid (TCA) served as deproteinizing agent. In certain cases, the filtrate was assayed for 2-pyrrolecarboxylic acid by addition of *p*-dimethylaminobenzaldehyde (PDAB); in others, the filtrate was extracted with ether so as to remove the TCA and the 2-pyrrolecarboxylic acid. Aliquots of the extracted aqueous solution were chromatographed on paper and the distribution of the radioactivity on each chromatogram was established.

*2. In vivo Experiments*

Young male rats of the Wistar strain were used. Each animal was given, by intraperitoneal injection, a 1.5 ml. solution (pH 7.4) of 10 to 20 mgm. of labelled or normal hydroxyproline. The rats were killed by ether, 15, 60, min. or 24 hr. after injection. In the latter experiment, the 24-hr. urine was collected. The liver and the kidney tissues were homogenized separately in 5 ml. of water and an equal volume of 20% TCA was added. The filtrate was extracted with ether and aliquots of the aqueous solution were analyzed by paper chromatography.

*3. Paper Chromatographic and Radiochromatographic Techniques*

One-dimensional chromatograms were developed by capillary ascent on Whatman No. 1 filter paper, using the solvent systems described in Tables I and II. The amino acids were visualized by reaction with ninhydrin according to the usual procedure. Hydroxyproline and proline were also revealed by the use of isatin reagent (0.4% isatin and 4% acetic acid in *n*-butanol), which produces a blue color. To reveal the presence of 2-pyrrolecarboxylic acid, a 2% solution of PDAB in 1 *N* hydrochloric acid was employed. A characteristic red color develops rapidly upon warming.

The distribution of radioactivity on the chromatograms containing $C^{14}$-labelled metabolites was determined by the routine procedure employed in this laboratory (2). The identity of the $C^{14}$-pyrrolic metabolite with synthetic 2-pyrrolecarboxylic acid was established by the use of admixture paper chromatography. The radioactive peak due to the former coincided with the red spot due to the latter, which had been added in an amount such that it could be visualized with PDAB reagent.

*4. Synthesis of 2-Pyrrolecarboxylic Acid*

2-Pyrrolecarboxylic acid was prepared according to the method of Maxim *et al.* (3). The product was purified by crystallization from ether. Crystals of the pure substance melted with decomposition at 204–207°. Its purity was also ascertained by paper chromatography in many solvents.

## Results and Discussion

*1. Experiments with Rat Kidney and Liver Tissues*

Hydroxy-DL-proline-2-$C^{14}$ (1) was incubated in the presence of kidney homogenates. Paper chromatograms of the deproteinized media were prepared, and the radioactivity distribution was determined. Two radioactive peaks were detected on each chromatogram. The $R_f$ values are given in Table I. Peak I was found to be about ten times as radioactive as peak II. The substance responsible for peak I was readily identified as hydroxyproline by admixture chromatography and color reaction with ninhydrin or isatin. The compound at peak II did not give any color reaction with ninhydrin, but it gave a red color with PDAB reagent. It was identified as 2-pyrrolecarboxylic acid by cochromatography with the synthetic compound in five solvent systems (Table II). Experiments carried out with rat liver homogenates indicated that the activity of the liver tissue for the formation of PCA from hydroxy-DL-proline is much less than that of the kidney.

The unequivocal demonstration that the PDAB-positive metabolite is identical with synthetic 2-pyrrolecarboxylic acid was obtained by the following

TABLE I

$R_f$ VALUES OF RADIOACTIVE PEAKS ON PAPER CHROMATOGRAMS

| Chromatographic solvents | Peak I, hydroxyproline | Peak II, 2-pyrrolecarboxylic acid |
|---|---|---|
| Phenol–water (4 : 1) | 0.65 | 0.58 |
| *n*-Butanol – acetic acid – water (15 : 3 : 7) | 0.19 | 0.88 |
| Ethanol–water (77 : 23) | 0.40 | 0.74 |

TABLE II

COMPARISON OF THE $R_f$ VALUES OF THE PDAB-POSITIVE METABOLITE AND 2-PYRROLECARBOXYLIC ACID IN A VARIETY OF CHROMATOGRAPHIC SOLVENT SYSTEMS

| Solvent systems | $R_f$ values | | |
|---|---|---|---|
| | PDAB-positive metabolite | Synthetic 2-pyrrole-carboxylic acid | Admixture of both compounds |
| Phenol–water (4 : 1) | 0.58 | 0.59 | 0.58 |
| *n*-Butanol – acetic acid – water (15 : 3 : 7) | 0.88 | 0.90 | 0.88 |
| Ethanol–water (77 : 23) | 0.74 | 0.74 | 0.73 |
| *n*-Butanol – pyridine – water (5 : 5 : 2) | 0.57 | 0.58 | 0.57 |
| *s*-Collidine saturated with water | 0.44 | 0.45 | 0.43 |

experiments. Several incubations of normal hydroxy-DL-proline were made in the presence of rat kidney homogenates. The incubated media were pooled and the proteins were coagulated by prolonged heating and then separated by centrifugation. The supernatant solution was made alkaline to pH 10–11 with sodium hydroxide and then extracted twice with ether. The aqueous solution was acidified to pH 1–2 with hydrochloric acid and extracted with three portions of ether. The ethereal extract was dried over anhydrous sodium carbonate and then evaporated to dryness under reduced pressure. The residue contained the PDAB-positive metabolite.

A portion of the crude product was dissolved in a mixture of ethanol and acetic acid and the solution was treated with hydrogen at atmospheric pressure, in the presence of Adams catalyst. The formation of proline was confirmed by admixture chromatography in phenol–water, *n*-butanol – acetic acid – water, and ethanol–water.

For the purpose of determining the absorption curve of the metabolite under ultraviolet light, a portion of the crude preparation was dissolved in water and the solution was extracted with ether. The ethereal extract was evaporated, and the residue was dissolved in a small amount of water. The PDAB-positive compound was then purified by paper chromatography, using as solvent *n*-butanol – acetic acid – water. A 1 cm. paper strip was cut horizontally through the dried chromatogram, at the level of $R_f$ 0.90 and elution was made with a small volume of bidistilled water. The ultraviolet absorption spectra of both the compound contained in the eluate and synthetic PCA were identical, with a maximum at 256 m$\mu$. Furthermore, maximum absorption of light at 494 and 565 m$\mu$ was found for the colored product formed from both compounds by reaction with PDAB.

Carrying the biological investigation further, allohydroxy-DL-proline was incubated in the presence of rat kidney homogenates and found to be a precursor of PCA. However, color determinations indicated that nearly twice as much PCA originated from allohydroxy-D-proline than from the DL-mixture. Of special interest is the observation that no PCA was formed by incubating hydroxy-L-proline in the presence of liver or kidney homogenates.

If one considers the facts that hydroxy-L-proline does not give rise to PCA and that, on a weight basis, allohydroxy-D-proline generates twice as much PCA as the racemic mixture, one logically arrives at the conclusion that the D-hydroxyprolines, but not the L-isomers, are effectively the precursors of the pyrrolic compound.

The formation of PCA was also observed when slices of fresh rat kidney or liver tissues were incubated in a Krebs–Ringer phosphate buffer saturated with air or oxygen, in the presence of either one of the following substrates: hydroxy-DL-proline-2-$C^{14}$, hydroxy-DL-proline, allohydroxy-DL-proline, and allohydroxy-D-proline. In this series of experiments, the kidney tissue invariably showed more activity than the liver, as judged by the intensity of the color reaction of 2-pyrrolecarboxylic acid with PDAB. In the case

of the incubation experiment of hydroxy-DL-proline-2-$C^{14}$ carried out in the presence of liver slices, the latter were homogenized in water and the proteins were precipitated with TCA. The filtrate was extracted with ether to remove the TCA and the 2-pyrrolecarboxylic acid. Aliquots of the aqueous solution were chromatographed on paper with phenol–water as solvent mixture. An unidentified radioactive peak, representing about 4% of the total radioactivity on each chromatogram, was observed at $R_f$ 0.37.

*2. Experiments with Intact Rats*

A series of experiments were performed in which intact rats were injected intraperitoneally with hydroxy-DL-proline-2-$C^{14}$. Rat 1 weighing 100 gm. was given 20 mgm. of the labelled amino acid. To the 24-hr. urine was added as carrier 300 mgm. of synthetic PCA, and continuous ether extraction was carried out overnight. The dried ether extract was concentrated *in vacuo* and the crystals of PCA were collected and recrystallized in ether to constant radioactivity. It was found that about 4% of the injected radioactivity had been excreted in the urine in the form of PCA.

Two experiments with rats were carried out for a short period of time. Rats 2 and 3, each weighing about 50 gm., received 20 and 10 mgm. of hydroxy-DL-proline-2-$C^{14}$, respectively. Rat 2 was killed 15 min., and Rat 3, 60 min. after injection. The liver and kidney tissues of the animals were separately homogenized in water, using a Teflon-glass grinder. The homogenates were deproteinized with TCA and the filtrates were extracted with ether to remove the free TCA and the 2-pyrrolecarboxylic acid. Aliquots of the ether extracts were evaporated to dryness and the residues were taken up with water. A positive PDAB color test for 2-pyrrolecarboxylic acid was observed. On the other hand, aliquots of the ether-treated aqueous extracts (protein-free) of kidney and liver from Rats 2 and 3 were chromatographed on paper, using phenol–water as developing solvent system. Three distinct radioactive peaks were located on the paper chromatograms (Table III). A small but significant radioactivity was measured at $R_f$ 0.20, and the compound responsible for this peak (I) was identified as glutamic acid by admixture chromatography. The metabolite at peak II ($R_f$ 0.37) is as yet of unknown nature, while the major radioactive peak (III) was due to hydroxy-

TABLE III

RADIOCHROMATOGRAPHIC DATA FOR THE ETHER-TREATED AQUEOUS EXTRACTS (PROTEIN-FREE) OF LIVER AND KIDNEY OF RATS INJECTED WITH HYDROXY-DL-PROLINE-2-$C^{14}$

| Rat No. | Duration of experiment, min. | Tissue | $C^{14}$ content of extracts, expressed as % of the dose injected | Radioactivity distribution on each chromatogram (developed in phenol–water), expressed as % of the total radiocarbon measured | | |
|---|---|---|---|---|---|---|
| | | | | Peak I ($R_f$ 0.20), glutamic acid | Peak II ($R_f$ 0.37), unknown | Peak III ($R_f$ 0.65), hydroxyproline |
| 2 | 15 | Liver | 2.5 | 2 | 9 | 85 |
| | | Kidney | 2.0 | 6 | 2 | 91 |
| 3 | 60 | Liver | 3.5 | 2 | 14 | 84 |
| | | Kidney | 4.0 | 2 | 5 | 93 |

proline. The substance at peak II which is, from all evidence, the same as that already observed on chromatograms of liver slices extracts, was further examined by chromatography. The combined eluates of narrow paper strips, cut from large size chromatograms at the level of $R_f$ 0.37, were concentrated to a small volume. Aliquots were deposited on paper strips which were then chromatographically developed in three solvent mixtures. Each chromatogram showed a single radioactive peak at $R_f$ 0.21 in *n*-butanol – acetic acid – water, 0.58 in ethanol–water, and again 0.37 in phenol–water. Since the unknown compound had in the latter solvent the same $R_f$ value as that recorded before, it is obvious that it remained intact through the treatment of elution and rechromatography. It is seen from the results given in Table III that this unknown metabolite is formed particularly in the liver tissue.

To complete the series of experiments with rats, large doses (25 to 100 mgm.) of normal hydroxy-L-proline were administered to intact animals. In no case was the characteristic red color observed in the 24-hr. urine samples treated with PDAB, while a positive test was given by the urine of rats injected with hydroxy-DL-proline. The presence of 2-pyrrolecarboxylic acid in the urine samples giving a positive PDAB color test was confirmed by admixture chromatography with the synthetic compound.

*3. Experiments with Human Subjects*

It became interesting to find out if 2-pyrrolecarboxylic acid can be formed from hydroxy-DL-proline in the human organism as well. Three young men ingested late in the evening 1 gm. of the non-radioactive amino acid and the morning urine was collected. The urine samples were extracted with ether, the ethereal extracts were evaporated to dryness, and the residue was taken up with a small volume of PDAB reagent. Upon warming, an intense red color characteristic of PCA developed in each solution. A positive test was also given by the urine of human subjects who had ingested allohydroxy-D-proline. Experiments with natural hydroxyproline gave negative tests.

## Conclusion

The *in vivo* and *in vitro* studies described in the present communication justify the conclusion that there exists in the human and in the rat organisms an enzyme system which attacks hydroxy-D-proline and allohydroxy-D-proline to form 2-pyrrolecarboxylic acid. The results obtained with rat tissue slices and tissue homogenates indicate that, under comparable conditions, the kidney is more active than the liver in the production of 2-pyrrolecarboxylic acid. However, hydroxy-L-proline does not give rise to this pyrrolic substance. In view of the fact that neither hydroxy-D-proline nor allohydroxy-D-proline exist in mammalian tissues, the role of the enzyme system which favors the oxidation of these compounds is of completely unknown significance.

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# ACTIVATION OF SCHRADAN BY MAMMALIAN TISSUE HOMOGENATES[1]

By R. D. O'Brien[2]

## Abstract

It has been confirmed that the combined microsomal and soluble fractions of liver, in the presence of magnesium, nicotinamide, and DPN, can convert schradan to a potent anticholinesterase. DPN can be replaced by TPN, but not by ATP. DPNH can replace the DPN and soluble fraction. Catalase enhances the conversion but only when DPNH or a source of it is provided. Acetone powders of microsomes, suitably fortified, are almost ineffective in converting schradan. The pH optimum of the whole schradan-converting system is 8.1. Other properties of the system are described. Certain extrahepatic tissues, especially lung, heart, and testis, can convert schradan. The livers of all species tested convert schradan. A peroxide-mediated mechanism of oxidation is proposed.

The insecticide schradan (octamethylpyrophosphoramide) is readily converted to a powerful anticholinesterase ("activated") by mammalian liver slices (8) and by several whole tissues from all the insects so far studied (15). Homogenates of liver (9) or of insect tissues (15) are ineffective converters in the absence of added cofactors, or with the cofactors investigated previously (4, 16). Davison (7) has now shown that the microsomes plus supernatant fluid of rat liver homogenates can convert schradan in the presence of added magnesium, DPN,[3] and nicotinamide. This paper presents a study of the properties and distribution of the activating system in mammals.

## Methods

One milliliter of 0.067 *M* phosphate buffer pH 7.4 (or other buffer where indicated) and 0.5 ml. of a cofactor solution arranged to give final concentrations of $MgCl_2$ 0.015 *M*, DPN or TPN $4 \times 10^{-4}$ *M* (Sigma 95% preparations), and nicotinamide 0.008 *M* were placed in the Warburg flask with 1 ml. of human plasma which had been diluted 2.5 times. Tissue in 0.9% KCl was homogenized in a plastic-pestle Potter–Elvehjem type homogenizer at room temperature, and 0.5 ml. of the homogenate added to the flask. (In early experiments, preceding the temperature-effect experiments described below, an ice-cooled homogenizer was used.) In the acetone powder preparations, the hog or rat liver was homogenized in an iced Waring blendor for 6 min., 30 sec. on and 30 sec. off alternately. To the side-arm was added 0.2 ml. of 5% acetyl choline bromide, then at zero time 0.25 ml. of 0.5% schradan (prepared as in (4)) was added to the main compartment. The flasks were shaken gently on the platform of a Burrell wrist-action shaker for 50 min., then

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[3]*The following abbreviations will be used: DPN, diphosphopyridine nucleotide; DPNH, reduced DPN; TPN, triphosphopyridine nucleotide; ATP, adenosine triphosphate.*

removed and 0.3 ml. of 2.52% $NaHCO_3$ added to the main compartment. The flasks were now placed on their manometers, placed in the bath at 25°, and gassed (starting at 60 min. from zero) for 5 min. with 5% $CO_2$ in $N_2$. At 90 min. from zero the manometers were closed, the flasks were tipped, and readings begun. The volume of $CO_2$ released in 30 min. was calculated statistically from four readings by the method of Albridge, Berry, and Davies (2).

The method involves simultaneous conversion of schradan to the active compound by the added tissue, and the inhibition of the serum cholinesterase by this compound. At the concentrations used, schradan itself is ineffective as an anticholinesterase. After correction for the effects of cofactors, etc., upon the plasma cholinesterase, the per cent inhibitions of cholinesterase were used to calculate the micromoles of schradan activated, using the concentration–activity curve previously published for the active anticholinesterase derived from permanganate oxidation of schradan (16); 50% inhibition is given by $4 \times 10^{-7}$ *M* inhibitor. The identity of the anticholinesterases from permanganate oxidation and liver conversion has been shown (17). Unless otherwise stated, the final concentrations are given and a different tissue sample was used for each experiment.

Centrifugations were made in the cold room (2° C.) in a model SS2 Servall vacuum centrifuge. Mitochondria and heavier particles were brought down by spinning at 10,000 × *g* for 10 min., microsomes by 20,000 × *g* for 90 min. (or in the later work, 30,000 × *g* for 30 min.). The supernatant referred to in the paper is that remaining after the microsomes are spun down. Microsomes were washed once, taken up in 0.9% KCl, and gently homogenized by hand in a chilled all-glass Potter–Elvehjem homogenizer.

Acetone powders were prepared by pouring the microsome suspension (about 10%) slowly into 10 volumes of acetone at −15° C. with stirring, letting the mixture stand 10 min., filtering off the precipitate, and washing the precipitate once (acetone, −15° C.). The temperature never rose above −12° C. Powders were kept desiccated and frozen. The acetyl choline bromide was purified by ether precipitation of an ethanolic solution of the Eastman Kodak product. Protein determinations were made by the Folin–Ciocalteu method, standardized against blood albumin.

## Results

### *The Effect of Various Factors on Schradan Activation*

The findings of Davison (7) for rat liver were confirmed for the mouse, (Expts. 1, 2, and 3, Table I). For effective conversion the cofactors $Mg^{++}$, DPN, and nicotinamide are required. Both microsomes and supernatant are needed for full activity, but the supernatant is not solely a source of (heat-stable) cofactors, as boiling it removes its activity.

Experiment 4 shows that DPNH can replace the supernatant. The significance of this will be commented upon in the discussion. The control shows that this effect is not due to an increase in DPN following oxidation of the

TABLE I

SCHRADAN CONVERSION BY MOUSE LIVER

| | % cholinesterase inhibition | Micromoles schradan activated |
|---|---|---|
| Experiment 1 | | |
| Whole liver | 100 | >60 |
| Whole liver, no $Mg^{++}$ or DPN or nicotinamide | 8 | 0.17 |
| Experiment 2 | | |
| Whole liver | 95 | 20 |
| Whole liver, no $Mg^{++}$ | 73 | 3.98 |
| Whole liver, no DPN | 14 | 0.29 |
| Whole liver, no nicotinamide | 80 | 5.66 |
| Experiment 3 | | |
| Microsomes | 59 | 2.16 |
| Microsomes + supernatant | 100 | >60 |
| Microsomes + boiled supernatant | 58 | 2.06 |
| Supernatant | 28 | 0.62 |
| Experiment 4 | | |
| Microsomes | 30 | 0.67 |
| Microsomes + DPNH | 72 | 3.75 |
| Microsomes + extra DPN | 25 | 0.53 |
| Microsomes + old supernatant | 78 | 5.06 |
| Old microsomes + supernatant | 75 | 4.41 |
| Microsomes + dialyzed old supernatant | 59 | 2.16 |
| Microsomes + supernatant, TPN in place of DPN | 99 | 56.8 |
| Freezer-stored whole liver | 89 | 10.1 |
| Experiment 5 (0.5% liver) | | |
| Whole liver | 77 | 4.84 |
| Whole liver, less DPN | 54 | 1.76 |
| Whole liver, less nicotinamide | 89 | 10.1 |
| Whole liver, less Mg | 60 | 2.26 |
| Experiment 6 (1% liver) | | |
| Microsomes, supernatant | 43 | 1.17 |
| Microsomes, supernatant + catalase | 59 | 2.16 |
| Microsomes, supernatant, no DPN | 31 | 0.71 |
| Microsomes, supernatant + catalase, no DPN | 35 | 0.83 |
| Experiment 7 (5% liver) | | |
| Microsomes, supernatant | 70 | 3.42 |
| Microsomes, catalase, no DPN or nicotinamide | 3 | 0.08 |
| Experiment 8 (1% frozen rat liver) | | |
| Microsomes, DPNH | 70 | 3.42 |
| Microsomes, DPNH + catalase | 79 | 5.36 |
| Whole liver | 52 | 1.64 |
| Whole liver + catalase | 78 | 5.06 |

NOTE: *Except where otherwise stated, conditions were: Homogenates of fresh mouse liver, 10% (1, 3) or 6% (4) initial concentration, whole or fractionated as shown. Microsomes unwashed. All have plasma, phosphate buffer, magnesium, nicotinamide, and DPN. DPNH and extra DPN at* $4 \times 10^{-4}$ *M. Catalase* $1.4 \times 10^{-5}$ *M. "Old" supernatant and microsomes stored three days at 3° C. "Freezer-stored liver" kept four months at −18° C.*

added DPNH. Microsomes and supernatant deteriorated somewhat in activity following storage in the refrigerator. Dialysis did not eliminate the activity of this stored supernatant, but reduced it, possibly owing to some denaturation during the exposure to hypotonic conditions. TPN was very effective as a substitute for DPN. Liver which had been stored frozen for months is still very active in schradan conversion.

Excellent maintenance of activity was found at quite low liver concentrations (see below) but under these conditions results were somewhat different from those obtained above. Nicotinamide had a slight inhibitory effect (Experiment 5) now that (presumably) the masking effect of the high DPN-ase concentration had been reduced. A corollary was the reduced need for added DPN, although it was still necessary for optimal activity.

The capacity of ATP to replace DPNH was studied. As microsomes contain a Mg-activated ATP-ase (1) fluoride is necessary for its inhibition. Fluoride had only a small effect upon the normal system. As the normal oxidation of DPNH produces 3 moles of high-energy phosphate, ATP was used at three times the DPNH concentration; considerable increase in cholinesterase inhibition was found, but control experiments showed that this could be attributed entirely to an inhibition of cholinesterase which occurs in the absence of the liver fractions. Further experiments showed that there are two effects involved. Schradan, ATP, and fluoride ($10^{-3}$ *M*) together inhibit cholinesterase substantially (20–25%); ATP, nicotinamide, and fluoride together inhibit cholinesterase 25–30%. Some of these effects are due to a direct inhibition by fluoride (11%). The effects are for liver-free systems and have been confirmed in five experiments.

The effect of catalase upon the system was studied in order to investigate the possibility of hydrogen peroxide being concerned in the schradan oxidation. Experiment 6 shows that catalase increases activation by the fortified liver system, but has only a small effect if DPN is omitted. Experiment 7 shows that catalase cannot replace the DPNH-producing system (i.e. nicotinamide, DPN, supernatant). Experiment 8 shows that the catalase enhancement is also found in the whole-liver system, and in the system where supernatant is replaced by DPNH.

*Large-scale Microsome Preparations*

As the supernatant fraction acts as a DPNH source, the actual schradan-converting system must be microsomal, and attempts were made to prepare a partially purified system starting with an acetone powder. Considerations of quantity suggested hog liver as a suitable starting material, and experiments were carried out to determine its suitability and (since the amounts used precluded the use of a Potter–Elvehjem homogenizer) the effect of various treatments upon its activity.

Table II, Experiment 9, shows that although whole hog liver is effective, the microsomes and supernatant together are very poor. As the Waring blendor had been used in this preparation its influence was studied. The

## TABLE II

SCHRADAN CONVERSION BY HOG AND RAT PREPARATIONS

| | | % cholinesterase inhibition | Micromoles schradan activated |
|---|---|---|---|
| Experiment 9 (using liver stored intact under 0.9% KCl, 2° C. 6 days) | | | |
| Whole liver | Homogenized in micro-Waring, 1 min. | 35 | 0.83 |
| Mitochondria + supernatant | | 33 | 0.78 |
| Microsomes + supernatant | | 8 | 0.17 |
| Supernatant | | 10 | 0.21 |
| Whole liver | Homogenized in Potter–Elvehjem, 2 min. | 51 | 1.57 |
| Mitochondria + supernatant | | 32 | 0.75 |
| Microsomes + supernatant | | 0 | 0 |
| Supernatant | | 1 | 0.04 |
| Experiment 10 (whole liver) | | | |
| Homog. in Potter–Elvehjem, ice-cold, 1 min. | | 73 | 3.98 |
| Homog. in Potter–Elvehjem at room temperature 1 min., left to stand 1 hr. at room temperature | | 73 | 3.98 |
| Homog. in iced micro-Waring 0.5 min. | | 61 | 2.34 |
| Homog. in iced micro-Waring 1.5 min. | | 65 | 2.78 |
| Homog. in iced micro-Waring 3 min. | | 15 | 2.91 |
| Homog. in iced micro-Waring 6 min. | | 15 | 2.91 |
| Experiment 11 | | | |
| Precipitate from 470 × *g* + supernatant from 1400 × *g* | | 48 | 1.40 |
| Precipitate from 760 × *g* + supernatant from 1400 × *g* | | 36 | 0.86 |
| Precipitate from 1400 × *g* + supernatant from 1400 × *g* | | 35 | 0.83 |
| Supernatant from 1400 × *g* | | 35 | 0.83 |
| Experiment 12 | | | |
| Precipitate from 470 × *g* – supernatant from 470 × *g* | | 52 | 1.64 |
| Precipitate from 470 × *g* | | 20 | 0.41 |
| Supernatant from 470 × *g* | | 37 | 0.90 |
| Experiment 13 (fresh 10% rat liver) | | | |
| Whole liver | | 97 | 30.0 |
| Microsomes + supernatant | | 84 | 7.16 |
| Supernatant | | 3 | 0.08 |
| Supernatant + 3 mgm. acetone powder of microsomes | | 6 | 0.14 |

NOTE: *Except where otherwise stated, conditions were: 10% (initial concentration) homogenates of frozen hog liver, whole or fractionated as shown. Cofactors ($Mg^{++}$, DPN, nicotinamide), plasma, phosphate buffer present in all. Mouse supernatant and microsomes as in Table I, Expt. 2, but stored 6 days at 2° C. Acetone powder, 1.1 mgm. equivalent to about 4 × amount of liver in other flasks. Whole liver was homogenized in Potter–Elvehjem homogenizer. The "mitochondrial" fraction includes all the heavier components also. 3 mgm. acetone powder is equivalent to 8 × concentration of whole liver.*

results show that even the whole liver is adversely affected by blendor treatment, and also that the supernatant is more active in this case by itself (presumably by disintegration of microsomes). In both cases the microsomal fraction had an inhibitory effect (30%) upon cholinesterase in the absence of schradan, a curious phenomenon which may be explained by the presence of endogenous inhibitors (Karczmar and Koppanyi (11), on the basis of similar fractionation, reported endogenous inhibitors in several mammalian tissues). Most important of all, the schradan-converting activity is found almost entirely in the "mitochondrial" fraction. It was also observed that this liver specimen, which had been stored intact in KCl at 2° C. and was in poor condition, was consistently less effective than liver stored at −18° C. A further experiment upon the conditions of homogenizing was carried out (Experiment 10) and confirmed the reduced effectiveness of blendor preparations, but also showed that neither the time in the blendor nor (in the case of the Potter–Elvehjem) the temperature was important. After this, all Potter–Elvehjem homogenates were prepared at room temperature.

Low-speed fractionation was now undertaken, using a small angle-head MSE centrifuge at room temperature. (The hog liver used in this, and later experiments, was less active because it had thawed and had stood overnight at room temperature when a freezer was accidentally disconnected.) Experiments 11 and 12 show that even the heavy fraction coming down at 470 $\times$ *g* contributed significantly to the total. There was no activity in the fractions between 470 and 1400 $\times$ *g*, as all the observed inhibition would be accounted for on the basis of the supernatant present. Unfortunately fractionation between 1400 $\times$ *g* and 10,000 $\times$ *g* (close to the minimum measurable speed on the Servall) was not possible. The experiments indicate quite clearly that in hog liver most of the activity lies in the fraction precipitated at 10,000 $\times$ *g*, and a good deal is even in the precipitate at 470 $\times$ *g*.

In contrast to the situation in hog liver, the precipitate from 1400 $\times$ *g* from mouse liver homogenate was completely inactive. However, the activity is by no means confined to the microsomes. The fraction precipitated at 10,000 $\times$ *g* had 10 times the total activity of the microsomes.

As activity was desired in a clear-cut fraction in order to attempt purification and as quantity of tissue was required, rat liver was used. Excellent activity was found in whole liver and in the combined supernatant and microsomal fractions. However, Experiment 13 shows that the most concentrated acetone powder preparations were almost completely ineffective. This means that the most obvious first step in the purification procedure appears to destroy the enzyme system. This aspect of the work was therefore terminated for the present.

*The Effect of Concentration, Buffer, and pH on Schradan Activation*

The effect of different buffers upon schradan activation became of interest when it was observed that phosphate buffers inhibit activation by certain insect tissues. Fig. 1 shows that in the case of mouse liver, phosphate is the best of the three buffers investigated except at low liver concentrations, where

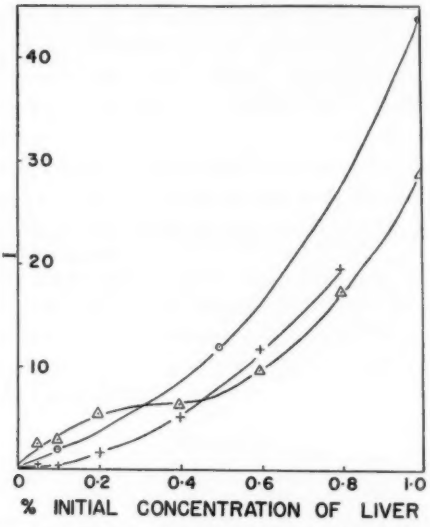



FIG. 1. The effect of liver concentrations on schradan activation in the presence of various buffers. Ordinate: $10^5 \times$ molar concentration of activated schradan. ○ Phosphate buffer. + Michaelis veronal buffer. △ Bicarbonate buffer (initial concentrations: $NaHCO_3$ $3.36 \times 10^{-2}$; NaCl $1.15 \times 10^{-1}$; KCl $2.4 \times 10^{-3}$; $CaCl_2$ $1.2 \times 10^{-3}$).

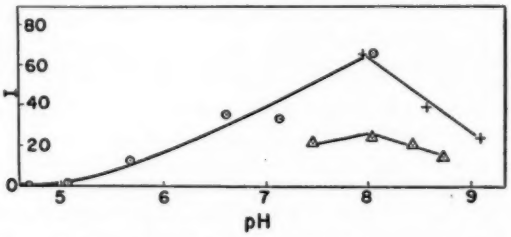



FIG. 2. The effect of pH on the activity of the schradan-converting system of mouse liver (fresh, 0.5% initial concentration). ○ Michaelis veronal buffer. + Glycine buffer. △ *Tris*(hydroxymethyl)aminomethane buffer. Ordinate: $10^5 \times$ molar concentration of activated schradan.

bicarbonate buffer is optimal, possibly owing to a sequestering effect of phosphate upon salts needed only in very small amounts, sufficient usually to be provided endogenously even in homogenates. This possibility is supported by the enhanced activity at low tissue concentrations with the bicarbonate buffer, which contained calcium and potassium.

The effect of pH upon the complete schradan-converting system was investigated using a low liver concentration (Fig. 2). In order to cover the whole range, veronal buffers were used for pH's up to 8, glycine–NaOH for pH's 8 to 9, and *tris*(hydroxymethyl)aminomethane for pH's 7.5 to 9. The pH's given are those of the mixture of buffer, plasma, etc. This is not the same as the pH of the buffer alone, because of the buffering activity of

the plasma. The optimum is seen to be at 8.1. As different liver samples were used for each buffer series, it is impossible to say whether the *tris*-(hydroxymethyl)aminomethane buffer had an inhibitory effect on the conversion as suggested by the figure.

TABLE III

THE CONVERSION OF SCHRADAN BY VARIOUS MAMMALIAN TISSUES

| Tissue | Condition | Animal | Sex | Protein (mgm./ml. in homogenate) | % cholinesterase inhibition | Micromoles schradan activated |
|---|---|---|---|---|---|---|
| Ovary | Fresh | Rat | ♀ | — | 0 | 0 |
| Pancreas | " | " | ♀ | — | 0 | 0 |
| Voluntary muscle | " | " | ♀ | — | 0 | 0 |
| Kidney | " | " | ♂ | 28.1 | 0 | 0 |
| Spleen | " | " | ♂ | 23.2 | 0 | 0 |
| Brain | " | " | ♂ | 17.3 | 2 | 0.06 |
| Liver | " | " | ♂ | 32.6 | 97 | 30.3 |
| Testis | Frozen | " | ♂ | 11.7 | 50 | 1.49 |
| Testis | " | Cat | ♂ | 14.7 | 31 | 0.71 |
| Small intestine | Fresh | Rat | ♀ | 11.6 | 14 | 0.29 |
| Small intestine | " | Mouse | ♀ | — | 13 | 0.27 |
| Stomach | " | Rat | ♀ | 9.0 | 28 | 0.62 |
| Lung | " | " | ♀ | 21.5 | 61 | 2.34 |
| Lung | Frozen | " | ♂ | 18.0 | 32 | 0.75 |
| Lung | " | Cat | ♂ | 15.9 | 42 | 1.11 |
| Heart | Fresh | Rat | ♀ | 27.5 | 49 | 1.45 |
| Heart | " | Field mouse | ♀ | — | 15 | 0.30 |
| Heart | Frozen | Cat | ♂ | 14.9 | 37 | 0.90 |

CONDITIONS: *10% homogenates (except liver, 0.5). All have phosphate buffer with $Mg^{++}$, DPN, nicotinamide.*

TABLE IV

THE CONVERSION OF SCHRADAN BY LIVERS FROM VARIOUS MAMMALS

| Animal | Sex | Homogenate % (initial) | Protein (mgm./ml. in homogenate) | % cholinesterase inhibition | Micromoles schradan converted |
|---|---|---|---|---|---|
| Field mouse | ♀ | 0.5 | 1.50 | 18 | 0.36 |
| Child (eight days old) | ♀ | 0.5 | 1.05 | 6 | 0.14 |
| | | 5.0 | 14.5 | 16 | 0.32 |
| White mouse (Swiss) | ♀ | 0.5 | 1.40 | 22 | 0.46 |
| Rat | ♂ | 0.5 | 1.26 | 35 | 0.83 |
| Hog | ? | 0.5 | 1.14 | 30 | 0.67 |
| Horse | ? | 0.5 | 1.90 | 27 | 0.59 |
| Cat | ♂ | 0.5 | 1.45 | 5 | 0.12 |
| Rabbit (fresh liver) | ♂ | 2.0 | — | 0 | 0 |
| Rabbit (frozen liver) | ♂ | 10.00 | — | 49 | 1.45 |

CONDITIONS: *(except as shown): All had phosphate buffer, DPN, $Mg^{++}$, nicotinamide, plasma. Animals were mature adults. Livers were frozen.*

*Schradan Activation by Various Tissues and Species*

A survey was made of the capacity of various tissues to convert schradan, with the results given in Table III. Contrary to expectation (see Discussion) it was found that several extrahepatic tissues are effective, although none to the same extent as liver. Lung, heart, and testis were capable of making substantial conversions; small intestine and stomach were moderately effective. All the other tissues tested were completely ineffective. Fresh rat tissues were used, and confirmatory experiments with tissues from other animals were made where substantial conversions were found.

The capacity of livers from various mammals to convert schradan was studied using frozen tissues. The results given in Table IV showed that the eight species studied all had livers capable of the conversion. Rat liver was most effective; poor conversions were obtained with livers of an eight-day child, cat, and especially rabbit.

## Discussion

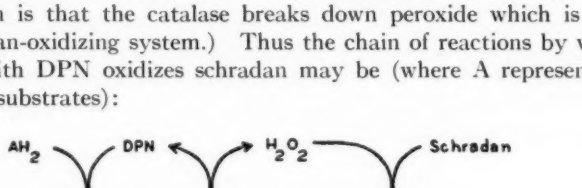
There are some important differences in these results compared with those of Davison (7). He found that TPN could not replace DPN, nor could DPNH replace the supernatant. The findings reported here have been consistently confirmed. Davison states that his TPN is "grossly impure", but does not give his DPNH source. Impurities may account for these discrepancies. He gives 7.3 as the pH optimum, compared with 8.1 in this study.

The conversion of schradan to the active anticholinesterase by tissues is oxidative (4, 8, 15) and it therefore seems surprising that DPNH (or a source of it, such as DPN with the glycolytic enzymes of the supernatant) is required. Cooper and Brodie (6) and Axelrod (3) also report systems oxidizing barbiturates and sympathomimetic amines by an apparently similar mechanism. These systems are microsomal and also have the odd requirement for reduced nucleotide, but in Cooper and Brodie's system DPNH cannot replace TPNH, nor can DPN replace TPN in the presence of the supernatant. The 4-dimethylaminoazobenzene demethylating enzyme of Mueller and Miller (14) used DPN (with magnesium and nicotinamide); ATP had a negligible effect when used either with or in place of DPN. It is not clear why Casida *et al.* (4) using such a technique failed to get fortification of liver homogenates with respect to schradan activation. Such specificity studies as are available suggest that the oxidation of diverse alkylamines are accomplished by diverse enzymes (3, 14) the most important similarity between the systems being a requirement for reduced pyridine nucleotide, magnesium, and oxygen.

The finding that ATP cannot replace DPNH at three times its concentration (assuming that 1 mole of DPNH can give rise to three energy-rich phosphate bonds (13)) added to the fact that the DPNH can be utilized in the absence of the mitochondria, suggests that schradan oxidation is not coupled to the normal DPNH–FAD–cytochrome system (13). The inhibitory effect of nicotinamide at low tissue concentrations also suggests that the DPNH is

being utilized by an enzyme other than those involved in normal oxidative phosphorylation, which are protected rather than inhibited by nicotinamide.

A possible mechanism is that DPNH is used to produce peroxide, through a suitable enzyme system, and this may oxidize the schradan either directly or enzymically. Such a mechanism might explain the effect of catalase upon the system; it was only effective in the presence of DPNH or a system capable of generating it. Catalase may be acting here as a peroxidase to catalyze the oxidation of schradan by peroxide produced in the system, as has been shown for ethanol oxidation (12). (A possible but unlikely alternative explanation is that the catalase breaks down peroxide which is inhibiting the schradan-oxidizing system.) Thus the chain of reactions by which liver fortified with DPN oxidizes schradan may be (where A represents various oxidizable substrates):




The association of activity in the hog liver with fractions heavier than the microsomal may be connected with the condition of the liver, which was fatty. Although Axelrod stated in an abstract (3) that ephedrine demethylase was "localized in the microsomal fraction" of liver, it is of interest that in the delivered paper, corresponding to this abstract, a slide showed that the nuclear fraction also had considerable activity (the ratio of activities of nuclear to microsomal fractions was 32 : 85).

The shape of the concentration–activity curves (Fig. 1) deserves comment. Possibly the reaction is inhibited by excess substrate (schradan) and this excess is lowered by the extra activity of the more concentrated liver preparation. Alternatively, there may be a factor present in the liver that is required in a certain minimum concentration for proper activity, but once this minimum is reached further increases in the concentration of the factor have no effect, and the concentration curve then tends to rise sharply and linearly.

Dubois, Doull, and Coon (8) showed that slices of kidney, cardiac muscles, and brain were ineffective in converting schradan. Cheng (5) showed indirectly that only the liver made the conversion, for schradan was nontoxic to the hepatectomized rat, and Frawley *et al.* (10) showed that carbon tetrachloride damage to liver raised resistance to schradan poisoning. The observation that other tissues than liver can convert schradan need not oppose such evidence; the liver is still seen to be by far the most important tissue in its capacity to convert very large quantities of schradan.

## Acknowledgments

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# THE DISTRIBUTION OF ADMINISTERED VITAMIN $K_1$-$C^{14}$ IN RATS[1]

By J. D. Taylor, G. J. Millar, L. B. Jaques, and J. W. T. Spinks

## Abstract

Radioactive vitamin $K_1$-$C^{14}$ was administered orally, intramuscularly, and intravenously to albino rats and the distribution of radioactive $C^{14}$ determined. Isotope dilution studies were performed to ascertain the degree to which the vitamin $K_1$-$C^{14}$ was present in unchanged form in some tissues. Regardless of the route of administration, radioactivity was deposited in all the tissues. The liver accumulated the largest amounts of radioactivity but the spleen had a much higher specific activity following intravenous administration of the vitamin. Radioactivity could be detected in the liver for as long as 12 days after administration. When the log of the dose of vitamin $K_1$-$C^{14}$ (5.6–82 mgm./kgm.) was plotted against the log of the vitamin $K_1$ equivalent in the various tissues a straight line relationship was obtained. The per cent of the injected dose present in the tissues 24 hr. after injection was as follows: liver 2.8, skeletal muscle 12.2, small intestine and contents 2.5, bile 26, feces 6.2, urine 0.98. The per cent of such activity present as unchanged vitamin $K_1$-$C^{14}$, as judged by isotope dilution tests, was liver 78, skeletal muscle 74, small intestine and contents 15, bile 1.5, feces 6.5, and urine 0.0.

Very little information is available at present on the levels of vitamin K in animal tissues. Dam and Schønheyder (6) showed that dried hog liver was a good source of vitamin K whereas chicken liver and cod liver were poor sources. Andrus, Lord, and Moore (1) showed a correlation between the corrective action of dried dog liver on the prothrombin time of K-deficient chicks and the prothrombin time of the bile–fistula dog from which the liver was taken. Greaves (11) reported that vitamin K was not stored in appreciable quantities in the liver of the rat. A vitamin K-free diet supplemented with 10% dried rat's liver failed to protect chicks from hemorrhage. Dam *et al.* reported that when massive doses of vitamin $K_1$ (100 mgm./kgm.) were administered to chickens and rats the unmetabolized vitamin $K_1$ could be extracted from the liver and spleen, and to a lesser extent the lungs and kidneys, 24 hr. after the administration of the vitamin (4). As the injection of India ink one day prior to the intravenous administration of vitamin $K_1$ in chicks caused retention of 12% of the injected dose in liver and 54% in spleen compared to values of 54% and 11% in the controls, it was suggested that the vitamin $K_1$ administered intravenously as a transparent colloidal solution (in tween 80) was deposited in the reticulo-endothelial system (5).

Recently several papers have appeared which indicate that the metabolism of vitamin $K_1$ might be different from vitamin $K_3$ (7, 15, 19). Chemically vitamin $K_1$ (2-methyl-3-phytyl-1,4-naphthoquinone) differs from vitamin $K_3$ (2-methyl-1,4-naphthoquinone) by having a long phytyl side chain (8). It has already been shown in this laboratory that the two vitamins differ

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*Contribution from the Department of Physiology and Pharmacology, University of Saskatchewan, Saskatoon, Saskatchewan. This work was supported by the National Research Council, Ottawa, Canada.*

quantitatively in their absorption from the gastrointestinal tract (16, 9) and also that the principal routes of absorption differ (16). In the present report it is shown, using $C^{14}$-labelled vitamin $K_1$, that the distribution, fixation in tissues, and excretion differ from those previously reported from this laboratory for vitamin $K_3$.

## Methods

Albino rats were used throughout these experiments. Radioactive vitamin $K_1$ (2-methyl-$C^{14}$-3-phytyl-1,4-naphthoquinone) was synthesized using a method developed by Dr. Otto Isler of F. Hoffman-LaRoche, A. G., Basle, (14, 17). The vitamin $K_1$-$C^{14}$ was administered as a transparent colloidal suspension in "tween 80" (2, 4), using light ether anesthesia by intravenous injection into the jugular or tail vein, or by intramuscular injections into the left hind leg, or by force feeding with a polyethylene stomach tube. The animals were sacrificed by decapitation after a period of time varying from five hours to 12 days. The organs were removed, dried to constant weight, ground in a mortar to a powder, plated on aluminum planchets, and assayed for $C^{14}$ content in a Chalk River type gas flow counter. Corrections were made for self-absorption to obtain the specific activity of the dry tissue in counts/min./mgm. The specific activity of the vitamin $K_1$-$C^{14}$ in counts/min./mgm. was obtained by plating a small aliquot of the suspension in an aluminum planchet, just prior to injection. It was then dried and counted in the manner given above. When radioactive vitamin $K_1$-$C^{14}$ has been administered to a rat the extent of metabolism of the vitamin at any given time is unknown and therefore it is convenient to speak of the vitamin $K_1$-$C^{14}$ equivalent of a given tissue. The vitamin $K_1$-$C^{14}$ equivalent was calculated from the specific activity of the dry tissue and the specific activity of the injected vitamin as follows:

Vitamin $K_1$-$C^{14}$ equivalent ($\mu$gm./gm.) =

$$\frac{\text{Specific activity of dry tissue (counts/min./mgm.)}}{\text{Specific activity of vitamin } K_1\text{-}C^{14} \text{ (counts/min./mgm.)}} \times \frac{10^6 \ \mu\text{gm.}}{1 \text{ gm.}}.$$

This manner of expressing the concentration of radioactivity in tissues has the added advantage of permitting a direct comparison when the specific activities of the vitamin $K_1$-$C^{14}$ preparations differed from one another.

Colorimetric assays for vitamin $K_1$ were carried out as described by Irreverre and Sullivan (13) except that 1 ml. of benzyl-trimethylammonium hydroxide was substituted for the 2% sodium alcoholate. Vitamin $K_1$ gives a blue color while vitamin $K_3$ yields a pink color. The blue color is not stable. It reaches a maximum intensity three minutes after the reagents have been mixed, after which it fades.

Vitamin $K_1$, vitamin $K_3$, and extracts of liver were chromatographed, using reversed phase partition chromatography, on siliconed Whatman No. 1 filter paper, according to the method described by Green and Dam (12). Ethyl alcohol: acetic acid: water (750 : 25 : 225) was employed as the solvent system.

## Results

To check the possibility that the radioactive methyl group of the vitamin $K_1$-$C^{14}$ might be metabolized to $CO_2$, a 156 gm. female rat was given a parenteral injection of 6.9 mgm./kgm. of radioactive vitamin $K_1$-$C^{14}$, specific activity $3.0 \times 10^5$ counts/min./mgm. The expired air was passed through sodium hydroxide for five hours and the carbon dioxide was precipitated as barium carbonate, which was washed, dried, weighed, and the $C^{14}$ activity assayed in the manner described above. The barium carbonate was found to contain no significant amount of radioactivity, e.g. specific activity $0.097 \pm 0.087$ (S.D.) counts/min./mgm. As little as 0.05% of the injected dose could have been detected.

Blood was obtained by carotid cannulation from two pairs of rats following oral administration of 3.0 mgm./kgm. of vitamin $K_1$-$C^{14}$, specific activity $1.33 \times 10^5$ counts/min./mgm. Plasma erythrocytes and buffy coat were obtained by centrifugation in plastic cups. The cells were washed twice with 0.9% NaCl and the plasma was fractionated by the method of Cohn *et al.* (3). Two rats were bled one hour after the vitamin was given and the other pair were bled at 16 hr. The vitamin $K_1$ equivalent (counts/min./100 ml. of blood) of the various fractions is given in Table I. One hour after oral ingestion of vitamin $K_1$-$C^{14}$ 69% of the activity present in the blood was in the plasma. The fraction which contained fibrinogen had a very high specific activity compared with the other fractions. The

TABLE I

A COMPARISON OF THE VITAMIN $K_1$-$C^{14}$ EQUIVALENT IN PLASMA, ERYTHROCYTES, AND BUFFY COAT FOLLOWING ORAL AND INTRAVENOUS ADMINISTRATION OF VITAMIN $K_1$-$C^{14}$

| | Time interval to collection of blood sample | | |
|---|---|---|---|
| | 1 hr. | 16 hr. | 5 hr. |
| | Vitamin $K_1$-$C^{14}$ equivalent, μgm./100 ml. blood | | |
| | Oral | | Intravenous |
| Plasma | 68 | 28 | 53 |
| Washed erythrocytes | 31 | 31 | 27 |
| "Buffy coat" | 0.34 | 2.8 | 3.3 |
| Plasma fractions: | | | |
| I + III—3 | 35 | 0.97 | |
| II | 4.0 | N.A. | |
| III—0 | N.A. | N.A. | |
| III—1, 2 | 1.0 | N.A. | |
| IV—1 | 2.9 | 0.75 | |
| IV—6 + 7 | 1.2 | 0.50 | |
| V | 6.2 | 3.7 | |
| VI | 2.7 | 3.3 | |

NOTE: *Oral dose of $K_1$-$C^{14}$ = 3.0 mgm./kgm. (specific activity $1.33 \times 10^5$ counts/min./mgm.). Intravenous dose of $K_1$-$C^{14}$ = 6.0 mgm./kgm. (specific activity $3.0 \times 10^5$ counts/mim./mgm.).*

*N.A. = no significant activity.*

level of $C^{14}$ in the 16 hr. plasma was only 25% of the radioactivity present in the whole blood. The vitamin $K_1$ equivalent of the washed erythrocytes was the same after 16 hr. whereas that of the buffy coat had increased approximately eightfold. The level of radioactivity had decreased in all the plasma fractions but the greatest decrease was in the fibrinogen-containing fraction (I + III − 3). The albumin-containing fractions IV, V, and VI carried most of the activity in the plasma after 16 hr. On analysis of the blood from the rat described in the first paragraph, five hours after intravenous administration of 6.9 mgm./kgm. of vitamin $K_1$-$C^{14}$, specific activity $3.0 \times 10^5$ counts/min./mgm., the plasma contained about 64% of the total activity present in the whole blood. On a dry weight basis the buffy coat had a specific activity of $3.78 \pm 0.102$ counts/min./mgm. compared with $0.63 \pm 0.83$ counts/min./mgm. for the erythrocytes. The erythrocytes however, because of their greater weight, had a greater vitamin $K_1$ equivalent (counts/min./100 ml. of blood).

A preliminary investigation of the distribution of radioactive vitamin $K_1$ was conducted as follows: A 310 gm. female rat was given an intravenous injection of 0.32 mgm./kgm. of vitamin $K_1$-$C^{14}$, specific activity $3.0 \times 10^5$ counts/min./mgm. Nine hours later the liver, lung, kidney, spleen, heart, large bowel and contents, small intestine and contents, stomach and contents, and the urine were assayed for $C^{14}$ content. The rest of the animal was run through a meat chopper and treated as above. The results presented in Table II show the distribution of radioactivity in this rat. All the radioactivity injected was accounted for. The liver appears to concentrate vitamin $K_1$ even at doses of less than 1 mgm./kgm. of body weight. The lung and the spleen also accumulated some radioactivity. It is also apparent

TABLE II

DISTRIBUTION OF RADIOACTIVITY IN A RAT NINE HOURS AFTER THE INTRAVENOUS ADMINISTRATION OF VITAMIN $K_1$-$C^{14}$

| Organ | % of injected activity deposited in the organ | Vitamin $K_1$-$C^{14}$ equivalent, μgm./gm. tissue |
|---|---|---|
| Liver | 16.60 | 6.07 |
| Lung | 1.75 | 4.80 |
| Kidney | 0.23 | 0.37 |
| Spleen | 0.93 | 4.54 |
| Heart | 0.22 | 0.67 |
| Caecum, large bowel and contents | 4.30 | 3.50 |
| Stomach and contents | 0.38 | 0.90 |
| Small intestine and contents | 2.20 | 3.90 |
| Urine | 1.02 | — |
| Remainder | 79.00 | 1.13 |
| Total % | 107.0 | |

NOTE: *Weight of rat – 310 gm.*
*Dose of $K_1$-$C^{14}$ = 0.32 mgm./kgm. (specific activity $3.0 \times 10^5$ counts/min./mgm.).*

that activity was present in the gastrointestinal tract in fairly high concentration. The small intestine and its contents showed a higher specific activity than the caecum and large bowel and contents although both contained considerable activity. The caecum and large bowel and contents contained a greater percentage of the injected dose because of their larger bulk.

In order to determine whether the route of administration affected the deposition of vitamin $K_1$ in the tissues of rats, three female rats weighing 144, 155, and 160 gm. were each injected with 1 mgm. of radioactive vitamin $K_1$-$C^{14}$, specific activity $3.0 \times 10^5$ counts/min./mgm. One rat received the colloidal suspension by stomach tube, a second animal was given an intramuscular injection, and the remaining rat received an intrajugular injection. After 24 hr. the animals were sacrificed and the tissues were assayed for $C^{14}$. Table III shows a comparison of the tissue levels of radioactivity found in these animals. At a dose of 6–7 mgm./kgm. it would appear that there is very little difference in the vitamin $K_1$-$C^{14}$ equivalent deposited in the liver when the vitamin is administered by the various routes. The vitamin $K_1$-$C^{14}$ equivalent of the blood and heart of the rat given an intravenous injection was lower than those of either of the other animals. On the other hand the spleen of the animal given vitamin $K_1$ intravenously had a higher vitamin $K_1$-$C^{14}$ equivalent than did those of the other animals. It should be pointed out that a large amount of the vitamin $K_1$ given intramuscularly remained at the site of injection.

In order to study the duration of the retention of radioactivity in the tissues, six female rats were given 5.2 mgm. of vitamin $K_1$ in 5% tween 80. Three of these animals were given intravenous injections and three were given intramuscular injections. The animals were sacrificed at one, three, and

TABLE III

COMPARISON OF ORAL, INTRAMUSCULAR, AND INTRAVENOUS ADMINISTRATION OF VITAMIN $K_1$-$C^{14}$ ON THE VITAMIN $K_1$-$C^{14}$ EQUIVALENT AND PER CENT OF ADMINISTERED ACTIVITY IN RAT TISSUES

| Tissue assayed | Route of administration of vitamin $K_1$-$C^{14}$ | | | | | |
|---|---|---|---|---|---|---|
| | Intravenous | | Intramuscular | | Oral | |
| | Vitamin $K_1$-$C^{14}$ equivalent, μgm./gm. dry tissue | % of injected activity deposited in the tissue | Vitamin $K_1$-$C^{14}$ equivalent, μgm./gm. dry tissue | % of injected activity deposited in the tissue | Vitamin $K_1$-$C^{14}$ equivalent, μgm./gm. dry tissue | % of injected activity deposited in the tissue |
| Liver | 21.0 | 3.50 | 15.0 | 2.70 | 18.0 | 2.60 |
| Lung | 5.8 | 0.13 | 5.6 | 0.13 | 10.0 | 0.21 |
| Kidney | 6.1 | 0.16 | 3.7 | 0.11 | 4.8 | 0.15 |
| Spleen | 35.0 | 0.59 | 8.6 | 0.22 | 8.4 | 0.09 |
| Heart | 0.9 | 0.01 | 3.7 | 0.47 | 8.9 | 0.13 |
| Blood | 0.6 | 0.19† | 4.5 | 1.39† | 3.4 | 0.99† |
| Muscle | 0.4 | 1.72‡ | 130.0* | 19.00* | 2.5 | 9.64‡ |

* *Site of intramuscular injection.*
† *Calculated on the basis of blood constituting 2% of the dry weight of the rat's body.*
‡ *Calculated on the basis of skeletal muscle constituting 27% of the dry weight of the rat's body.*
*Dose—1.0 mgm. (specific activity $3.0 \times 10^5$ counts/min./mgm.).*

six days. The urine and feces were collected and pooled and the cumulative fecal and urinary excretions were determined. The results of this experiment are shown in Table IV. A progressive decrease of radioactivity in the livers of both groups of rats and an increased fecal and urinary excretion is evident. Radioactivity remained in the livers and spleens of both groups for as long as six days. The cumulative fecal excretions at six days exceeded the urinary excretion by a factor of two to three. The total excretion in six days amounted to 52% and 66% of the injected doses. When the radioactive vitamin was given intravenously, the liver and spleen retained appreciable amounts of radioactivity but when the vitamin $K_1$-$C^{14}$ was injected intramuscularly the spleen retained only a small amount of the radioactivity although the liver levels were similar to those for the intravenous injection. Approximately 30% of the intramuscular dose remained at the site of injection after 24 hr. and about 4% was still present after 144 hr.

In order to test the effect of the magnitude of the administered dose on the tissue deposition of radioactive vitamin $K_1$ five young female rats were each given an intrajugular injection of vitamin $K_1$ in tween 80. The amount of radioactive vitamin given each rat was 5 mgm./kgm. but the amount of

TABLE IV

COMPARISON OF THE VITAMIN $K_1$-$C^{14}$ EQUIVALENT AND THE PER CENT OF INJECTED ACTIVITY DEPOSITED IN TISSUES OF RATS 24, 72, AND 144 HR. AFTER INTRAVENOUS AND INTRAMUSCULAR ADMINISTRATION OF VITAMIN $K_1$-$C^{14}$†

| | | Route of administration of vitamin $K_1$-$C^{14}$ | | | |
|---|---|---|---|---|---|
| | | Intravenous | | Intramuscular | |
| Time of sacrifice (hr.) | Tissue assayed | Vitamin $K_1$-$C^{14}$ equivalent, μgm./gm. dry tissue | % of injected activity deposited in the tissue | Vitamin $K_1$-$C^{14}$ equivalent, μgm./gm. dry tissue | % of injected activity deposited in the tissue |
| 24 | Liver | 300 | 8.20 | 180 | 10.00 |
| | Lung | 80 | .33 | 90 | .72 |
| | Kidney | 50 | .28 | 84 | .67 |
| | Spleen | 2480 | 9.10 | 72 | .27 |
| | Heart | 52 | .18 | 63 | .24 |
| | Blood | 50 | 3.60* | 63 | 5.10* |
| | Injection site | | | 1080 | 32.00 |
| | Feces | 555 | 5.30 | 148 | 5.50 |
| | Urine | | 9.70 | | 3.70 |
| 72 | Liver | 98 | 3.30 | 96 | 5.00 |
| | Lung | 70 | .35 | 96 | .49 |
| | Kidney | 21 | .12 | 35 | .03 |
| | Spleen | 210 | .90 | 42 | .02 |
| | Heart | 85 | .21 | 49 | .02 |
| | Blood | 24 | 1.85* | 54 | 3.80* |
| | Injection site | | | 490 | 5.60 |
| | Feces | 250 | 12.00 | 185 | 26.00 |
| | Urine | | 17.00 | | 9.70 |
| 144 | Liver | 70 | 2.10 | 69.0 | 3.90 |
| | Lung | 21 | 0.13 | 1.0 | 0.01 |
| | Kidney | 26 | 0.12 | 1.6 | 0.01 |
| | Spleen | 290 | 1.35 | 4.7 | 0.02 |
| | Heart | 60 | 0.20 | 2.5 | 0.01 |
| | Blood | 13 | 0.95* | N.A.‡ | N.A.‡ |
| | Injection site | | | 480 | 4.40 |
| | Feces | 382 | 43.00 | 190 | 38.0 |
| | Urine | | 19.00 | | 14.0 |

* *Calculated on the basis of blood constituting 2% of the dry weight of a rat's body.*

† *The specific activity of the vitamin $K_1$-$C^{14}$ was $6.15 \times 10^8$ counts/min./mgm. for the intravenous injections and $5.70 \times 10^8$ counts/min./mgm. for the intramuscular injections. Dose was 26 mgm./kgm.*

‡*Radioactivity not statistically different from background.*

nonradioactive vitamin was varied to give the total amount of vitamin indicated in Fig. 1. The animals were sacrificed 24 hr. after the injection and the radioactivity in the livers, lungs, kidneys, spleens, hearts, blood, skeletal muscle, urine, and feces was determined. The amount of radioactivity deposited in all the tissues examined increased with increasing doses of radioactive vitamin $K_1$ but this increase was most marked in the case of the liver, heart, and spleen. Fig. 1 shows the calculated vitamin $K_1$ equivalent in $\mu$gm./gm. of dry tissue plotted against the dose of vitamin $K_1$ in mgm./kgm. both on log scales. As the size of the administered dose was increased the percentage of the dose deposited in the liver and the spleen tended to increase while the percentage excreted in the urine and feces tended to decrease.

Fig. 2 shows the values for the urinary and fecal excretion of radioactive vitamin $K_1$ plotted against the dose of vitamin $K_1$ in mgm./kgm. (both on logarithmic scales). The fecal excretion exceeded the urinary excretion for all the doses used. The large variation in fecal excretion can be accounted for on the basis of correspondingly large variations in the weight of the feces excreted by individual rats.

With the aid of reverse phase partition chromatography we were able to demonstrate the presence of unmetabolized vitamin $K_1$ in the liver of a 180 gm. female rat that had received a massive dose, 100 mgm./kgm. of vitamin $K_1$ in tween 80, by tail vein. This animal was killed four and one-half hours later and the liver was removed, ground with anhydrous sodium sulphate, and extracted with peroxide-free ether. The ether was evaporated *in vacuo* and the residue was extracted with absolute ethanol (4). After storage overnight at $-5°$ C. the solution was filtered and made to 10.0 ml. The vitamin $K_1$ content of the liver was estimated colorimetrically using the Irreverre–Sullivan reaction and a recovery of 22.8% of the injected vitamin

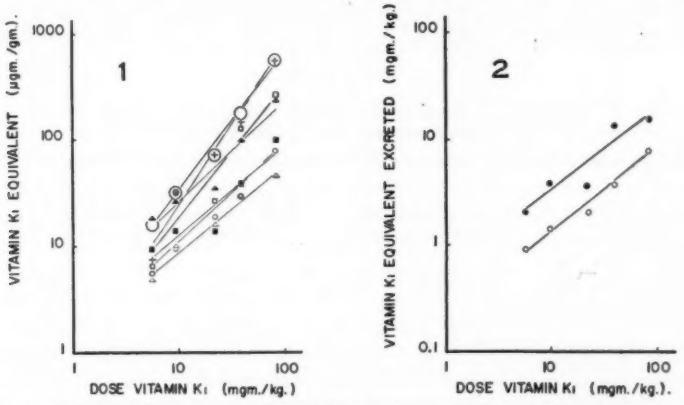


FIG. 1. Radioactivity of rat tissues following intravenous injection of vitamin $K_1$-$C^{14}$. ○—○ liver; ▲—▲ lung; ■—■ kidney; +—+ spleen; □—□ heart; ∘—∘ blood; △—△ muscle.

FIG. 2. Excretion of radioactivity following intravenous injection of vitamin $K_1$-$C^{14}$ in the rat. ●—● feces; ○—○ urine.

$K_1$ was attained. An aliquot containing 25 μgm. of vitamin $K_1$ was chromatographed simultaneously with control quantities of vitamin $K_1$ and vitamin $K_3$. After chromatography a spot was present which corresponded to a control spot of vitamin $K_1$ both in its position on the chromatogram and in its reaction to ultraviolet light.

The following is an example of the procedure and calculation employed in conducting the isotope dilution tests:

Approximately 27 gm. of wet muscle was removed from a 325 gm. male rat that had received, intravenously, 1 mgm. of vitamin $K_1$-$C^{14}$, specific activity $1.01 \times 10^6$ counts/min./mgm. The animal was sacrificed by decapitation 24 hr. after injection. The muscle was chopped, ground, and weighed; 2.06 gm. of wet muscle had a dry weight of 1.1052 gm. and a specific activity of 1.37 counts/min./mgm. (dry). On this basis the remaining 25.4 gm. of wet muscle would be equivalent to 13.6 gm. of dry muscle containing $1.86 \times 10^4$ counts/min. The 25.4 gm. of wet muscle was ground with 55 mgm. of vitamin $K_1$, 50 gm. of anhydrous sodium sulphate was added, and the mixture was ground to a powder and extracted with peroxide-free ether; the ether was removed *in vacuo*, and the residue was extracted with absolute ethanol. The alcoholic extract was refrigerated overnight at −5° C., filtered, extracted with petroleum ether (b.p. 30–60° C.), and chromatographed on permutit with petroleum ether, petroleum ether plus 10% benzene, and petroleum ether plus 20% benzene (20). The yellow oil that was obtained weighed 24.0 mgm. and assayed 24.4 mgm. using the Irreverre–Sullivan color reaction. It had a specific activity of 250 counts/min./mgm. The number of milligrams of labelled vitamin $K_1$-$C^{14}$ present in the muscle before the addition of carrier was $1.36 \times 10^{-2}$ mgm., i.e. 1.4% of the injected vitamin $K_1$-$C^{14}$. As the total activity in the sample before extraction was $1.86 \times 10^4$ counts/min. or 1.9% of the injected activity, it appears that approximately 74% of the activity in the muscle was present

TABLE V

ISOTOPE DILUTION TESTS ON RAT TISSUES FOLLOWING ADMINISTRATION OF VITAMIN $K_1$-$C^{14}$

| Tissue | % of injected $C^{14}$ present in sample | % of injected vitamin $K_1$-$C^{14}$ present in sample | % of activity in the tissue which is present as vitamin $K_1$-$C^{14}$ |
|---|---|---|---|
| Liver | 2.8 | 2.2 | 78 |
| Skeletal muscle | 1.9* | 1.4 | 74 |
| Small intestine and contents | 2.5 | 0.37 | 15 |
| Bile | 26.0 | 0.39 | 1.5 |
| Feces | 6.2 | 0.40 | 6.5 |
| Urine | 0.98 | 0.00 | 0.0 |

* *This corresponds to 12.2% of the injected activity in the total skeletal muscle on the basis of muscle comprising 27% of the dry weight of the rat's body.*

NOTE: *The liver was taken from one rat, the skeletal muscle, small intestine, feces, and urine from a second rat, while the bile was collected from a third animal. All received 1 mgm. of vitamin $K_1$-$C^{14}$ intravenously.*

as unmetabolized radioactive vitamin $K_1$-$C^{14}$. Isotope dilution tests were carried out on the other tissues in a similar manner and the results are presented in Table V. It can be seen that the liver contained 2.8% of the injected activity of which about 78% was present as unchanged vitamin $K_1$-$C^{14}$. The urine contained no unchanged vitamin $K_1$-$C^{14}$. The skeletal muscle presents a picture similar to that of the liver whereas the small intestine and its contents and the feces are intermediate. Bile was collected by fistula for 16 hr. and this contained 26% of the injected activity, but only 1.5% of the $C^{14}$ in the bile was present as unchanged vitamin $K_1$-$C^{14}$.

## Discussion

When radioactive vitamin $K_1$-$C^{14}$, labelled in the methyl group, was administered intravenously to a rat no significant amount of the radioactivity appeared in the expired air. It therefore appears that the 2-methyl-$C^{14}$ group was not removed and by inference the aromatic nucleus was not completely metabolized. A separation of the blood into plasma, erythrocytes, and buffy coat indicated that radioactivity could be detected in all three components. It would appear from our experiments that, in plasma, vitamin $K_1$ is associated with most of the protein fractions although the fibrinogen-containing fraction had the highest specific activity one hour after oral administration and the albumin-containing fractions carried most of the radioactivity after 16 hr. This shift in radioactivity from the fibrinogen to the albumin-containing fractions might be due to the metabolism of vitamin $K_1$-$C^{14}$. Since the prothrombin fraction from the 16 hr. plasma contained no significant activity it would appear that no appreciable amount of vitamin $K_1$-$C^{14}$ was incorporated into the prothrombin molecules. Solvonuk (18) obtained similar results following the administration of radioactive vitamin $K_3$-$C^{14}$. The high specific activity of the buffy coat compared to the erythrocytes could have been due to phagocytosis of the colloidal vitamin $K_1$ particles.

A comparison of the oral, intramuscular, and intravenous routes of administration showed that the vitamin was rapidly distributed throughout the body regardless of the route of administration. When vitamin $K_1$-$C^{14}$ was given intramuscularly, up to one third of the dose could still be found at the site of injection after 24 hr. The spleen accumulates more vitamin $K_1$ after intravenous injection than it does following either oral or intramuscular administration. Since radioactivity appeared in the bile soon after the administration of radioactive vitamin $K_1$-$C^{14}$ it was difficult to determine the completeness of absorption of the orally administered vitamin. The intravenous route was therefore chosen for a study of the effect of increasing doses of vitamin $K_1$. This experiment demonstrated that in all the tissues studied the deposition of the vitamin $K_1$ increased logarithmically as the log of the dose of the vitamin increased.

The isotope dilution test on the bile is of some interest because in the first place it confirms the observation (4) that no vitamin $K_1$ could be detected

in bile using the colorimetric assay of Irreverre and Sullivan while at the same time it shows that a "form" of the vitamin is present in the bile. Greaves (10) reported that bile itself contained low levels of an antihemorrhagic factor that would alleviate the prolonged prothrombin times of vitamin K deficient chicks. Furthermore, he was able to demonstrate that the active principle could be precipitated with zinc hydroxide and could be demonstrated after removal of the zinc.

Isotope dilution tests on urine and feces following intravenous administration of radioactive vitamin $K_1$-$C^{14}$ indicated that 100% of the activity in the urine was present as metabolites of vitamin $K_1$-$C^{14}$ and that only 6.5% of the activity in the feces was present in the form of the unchanged vitamin. Since only 1.5% of the radioactivity in the bile was unchanged vitamin $K_1$-$C^{14}$ while 15% of the activity found in the small intestine was present in this form, it appears that some reaction, such as hydrolysis of conjugates, had taken place. The form in which the metabolized vitamin $K_1$-$C^{14}$ exists in urine, feces, bile, and tissues has not been determined.

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# EXPERIMENTS ON THE USE OF SILICIC ACID FOR STEROID CHROMATOGRAPHY[1]

By J. H. Linford


## Abstract

Mixtures of pure sterols, and extracts of hydrolyzed urine, were chromatographed on columns of silicic acid. The results were observed spectrometrically using a sulphuric acid – methanol color reagent. Dry silicic acid retained or altered the sterols. When the silicic acid contained free water the sterols were distributed between the surface of the solid and the developing solvent. The adherence to the solid phase decreased with increase of free water and with increased solubility of the sterol in the solvent. As the free water was increased liquid–liquid partition was observed. The adherence of the sterol to the silica surface increased with the number of hydroxyl groups in the sterol molecule. Silicic acid columns are therefore particularly suited to the separation of sterols that differ in their number of hydroxyl groups and to the detection of the production of artifacts by loss of the hydroxyl group.


## Introduction

A comprehensive survey of steroid substances regularly found in human urine, and methods for their assay, has been published recently (7).

The work described below originated in the application of a sulphuric acid – alcohol reaction (5) to the detection of sterols in urine extracts. The reagent produces color with all sterols, but they may be distinguished by their visible region absorption spectra. The ultimate object was to detect neutral alcoholic and keto sterols, and phenolic sterols, after chromatographic separation. As measurements of absorbed light must be carried out on clear solutions and on a known length of the liquid if they are to be exact, column elution chromatography was adopted; the residues, after evaporation of the eluting solvent, were dissolved in the reagent. This method also permits isolation of quantities sufficient for subsequent examination.

Preliminary tests on the toluene extract of acid-hydrolyzed urine showed that a column of silicic acid retained the colored products of the acid hydrolysis in a sharper, narrower band than did alumina, calcium carbonate, or kieselguhr adsorbents. This was of importance because the reagent produces color with a majority of materials; the characteristic spectra of the sterols would be masked by an excess of extraneous absorption. It was therefore decided to first investigate the suitability of silicic acid as an adsorbent, using the available pure sterols and extracts of hydrolyzed urine as test materials.

## Reagents and Solvents

All reagents and solvents were of the highest available purity. Merck's reagent grade toluene and chloroform were used. "Analyzed reagent" grade methanol was obtained from Baker. "Analar" ethyl ether and "Analar" sulphuric acid were supplied by British Drug Houses. Before use the organic liquids were distilled in Pyrex just below the boiling point, with the exception of toluene; the latter overheats readily.

[1]*Manuscript received June 21, 1956.*
*Contribution from The Radiation Laboratories, The Manitoba Cancer Institute, Winnipeg, Man.*

## Silicic Acid Adsorbent

Methods for the laboratory preparation of silicic acid, to give particular adsorptive properties, have been described (3, 6). The application of silicic acid to steroid separation has been reviewed (2, 4). We purchased our material because we wished to have a larger quantity of one batch than we could prepare conveniently.

The adsorptive properties of silicic acid depend to a great extent upon the water retained both in its structure and on its surface (1). The structure is dependent upon such conditions as temperature and rate of mixing during the preparation. This affects the "bound" water; loss of this water by heating the acid at 1100° C. destroys the gel structure and the adsorptive properties. In addition the surface properties are a function of the "free" water present. The free water can be removed by heating the acid at 180° C. to 200° C. for an hour, or by washing it with certain solvents (8).

Samples were bought from different manufacturers, and samples of different lot numbers from the same manufacturer. The percentages of less than 80 mesh, 100 mesh, and greater than 200 mesh were found by sieving. The percentage of free water was measured by loss of weight on heating, and varied from 4% to 15% in different samples. For samples from the same batch, the use of coarser mesh was equivalent to use of a shorter column of finer mesh.

The free-water content was found to have a marked effect on the adsorption of sterols. This was controlled by enclosing samples of silicic acid in desiccators that contained solutions of known vapor pressure and were kept in a 25° C. air oven. The earlier experiments were carried out by enclosing 50 gm. lots of the adsorbent, in 10 cm. diameter Petri dishes, over 100 ml. of solutions made up of measured volumes of concentrated sulphuric acid and water. Later, 1.5 gm. lots of the silicic acid were weighed into crucibles, having a coarse sintered glass base, which were placed in separate desiccators over saturated solutions of calcium chloride hexahydrate and ammonium sulphate, and over solid anhydrous calcium chloride. The free-water content of the silicic acid attained an equilibrium with the vapor pressure of its atmosphere (Fig. 1). That a true equilibrium exists can be shown by transferring the sample to atmospheres of higher or lower vapor pressures. The curve then rises or falls with time to a new level.

By weight, the percentage increase in the dry weight due to uptake of free water at equilibrium at 25° C. was as follows: 17% over the calcium chloride solution; 32% over ammonium sulphate solution; 8% over a solution made of commercial sulphuric acid and water mixed in equal volumes. No change from the oven-dried weight occurred over anhydrous calcium chloride. Dry silicic acid also adsorbs the vapors of alcohol, toluene, and chloroform to approximately 50% by weight when it is enclosed over the pure liquid; this uptake in organic vapor is markedly reduced by the presence of free water. These organic solvents can be completely removed again by heating the acid at 200° C.

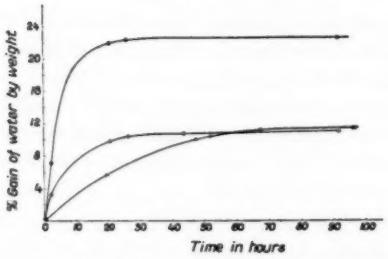


FIG. 1. Rate of increase of free-water content of silicic acid sealed in desiccators over water solutions: ●—●—, 5 gm. silicic over saturated ammonium sulphate solution; ○—○—, ×—×— 5 gm. and 10 gm. respectively over a solution made up of 40 ml. of concentrated sulphuric acid added to 60 ml. of water.

Experiments were carried out to determine whether the free-water content was decreased by the toluene–chloroform developer.

Free water is not removed from the silicic acid during four hours under a vacuum pump. Weighed samples containing known amounts of free water were washed with toluene, or with chloroform, and the organic phase was removed by a vacuum pump; the sample was reweighed, and the free-water content redetermined. From this we concluded that water is not removed from the silicic acid by these organic developers. Alcohol displaces water to a marked extent; the water is readily detected in the eluate.

It was found that the adsorption properties of the different samples could not be made uniform by introducing the same free-water content. The parameters by which the desired structural properties may be defined are not known; the adsorbents were therefore selected by their behavior with test substances. As a criterion we required, in toluene developer, that androsterone and dehydroisoandrosterone be separated from the dense pigment band characteristic of extracts of acid-hydrolyzed urine. Several samples were suitable; a sequence of samples of Mallinckrodt Acid Silicic, Analytical Reagent, were uniform in properties; therefore this was purchased in bulk.

## Detection and Measurement of Sterols

Sulphuric acid has been used as a color producing reagent for sterols by many workers (9). Modifications of the yellow color produced, by subsequent dilution sometimes coupled with further chemical reaction, are well known; they are often characteristic of individual steroids. Also the absorption shifts towards the red and thus to the region of less background absorption. Absorption measurements serve a dual purpose, as steroids in the color reagent can be detected and the quantities measured by the shape and height of the graphs of absorption spectra in the visible region.

For the present work, neutral steroids were dissolved in concentrated sulphuric acid by being heated at 55° C. for 1.0 hour. They were then diluted with an equal volume of methanol while being cooled in ice water.

The resultant solutions were stable; the absorption intensity changed 2% in three hours at room temperature. Methanol was selected as the dilutant since it produced a marked absorption in the red, maximum at 16,800 cm.$^{-1}$, with dehydroisoandrosterone and with certain urine components. The optical density followed Beer's law. Light absorption measurements within the region 14,000 to 25,500 cm.$^{-1}$ were made on a photoelectric spectrophotometer constructed in these laboratories.

Absolute identification of the sterols from urine has not yet been attempted. We propose to use infrared absorption for this purpose. Some sterols have been tentatively identified by analogy of their behavior with that of pure compounds on similar chromatographic columns, but displacement effects may occur.

## Chromatographic Methods

Hilger absorption cells of 1.00 cm. length, which required 2.5 ml. of solution, were initially used to contain the solutions of sterol in the color reagent. The steroid concentration that could be measured ranged from 5 to 100 μgm. per ml. of acid solution. This value governed the scale of the extraction and chromatographic procedures.

At a later stage in the experiments, the spectrophotometer was modified; the height of the spectrometer slits was cut to 2 mm. and a small lens of short focal length was mounted at the exit slit. A 1.00 cm. length absorption cell was then made from 6 mm. internal diameter tubing, which required only 0.3 ml. of liquid to fill. The scale of chromatography was then cut down by a factor of five.

In the initial experiments, the adsorbing material was contained in a glass chromatographic tube of 520 mm. length and 9 mm. inside diameter. This was fused to the bottom of a 125 ml. flask. With the use of the smaller cells in the spectrometer, the internal diameter of the column was reduced to 6 mm. The smaller column was jacketed by the outer sleeve of a West-type condenser and water at 25° C. circulated through it. The wider column held 5 gm. of silicic acid, the narrower 1.5 gm., to give a height of adsorbent of 12 cm. Pressure was applied to the column from a cylinder of nitrogen fitted with a diaphragm-type regulator. Toluene, chloroform, and ethanol were selected for trial as a representative series of eluents of increasing polarity.

The eluate from the larger column was usually collected in 5.0 ml. or 10.0 ml. fractions, from the smaller in 1.0 ml. or 2.0 ml. fractions. These were evaporated in lots of six under the vacuum produced by a water pump, in a water bath not warmer than 50° C. The residues were dissolved in Analar sulphuric acid, and diluted with reagent grade absolute methanol as described above.

To prepare a column the silicic acid was stirred into toluene in a beaker in the proportion of 1 gm. to 5 ml. This was poured into the column and followed by two rinses. To obtain even bands in the chromatogram it was found necessary to stir the suspension in the column and allow it to settle

under gravity for 15 min. Pressure up to 10 lb. per sq. in. was then applied and the column of wet adsorbent settled to a constant height. The pressure chosen was a few pounds higher than that at which the column was to be operated. The difference between the amount of liquid used to prepare the column, and that expressed when the miniscus just touched the adsorbent, gave the volume of the liquid held in the silicic acid. The extract to be chromatographed was added to the column and rinsed down with two lots of solvent; each volume was pressed into the column in succession. The developing fluid was then added; fractions of eluate were collected from the time of adding the extract.

## Results

*Chromatography of Pure Steroids*

Dehydroisoandrosterone (DHA) was chosen as the initial test steroid because of its lability and its ready detection by the acid–alcohol reagent. The criterion of adsorption was that the volume of eluent forced through the column before the appearance of the steroid, called the threshold volume, retention volume, or the forerun, was greater than the volume of liquid retained by the adsorbent. To allow for separation of faster running materials the greater the forerun, consistent with good recovery, the better.

The effect of the free-water content was marked. Using a developing mixture made up of equal volumes of toluene and chloroform, 100 μgm. of DHA could not be eluted from 5 gm. of silicic acid containing 5% of free water. Raising of the water content to 16% by allowing the acid to stand in a moist environment for 48 hr., or by adding liquid water, had no effect. Samples that contained 8% or more of free water when purchased, or which were raised to that value at equilibrium with a moist environment, yielded a good recovery, 75% to 92%. We concluded that in the samples wetted over a short term the wetting was not uniform and regions of drier acid existed. Intermediate conditions gave low recoveries, ranging from 7% to 60%. The forerun increased and the volume of eluent containing DHA increased with the drop in percentage recovery. We concluded that this represented a series of adsorption isotherms of increasingly steeper origin.

The use of sulphuric acid – water as a source of humidity for large batches of silicic acid, from which samples were repeatedly removed, produced successively drier samples. With 5 gm. columns of silicic acid in equilibrium with the sulphuric acid – water mixture, the forerun of DHA varied from 190 to 290 ml. and recoveries ranged from 75% to 90%. Using the saturated salt solutions to control humidity, and temperature-controlled columns with the 1.5 gm. lots of silicic acid, the forerun was constant to a milliliter and recoveries were 80 ± 2%.

Tests were carried out with silicic acid containing no free water; this acid had been stored over anhydrous calcium chloride after previous heating to 200° C. DHA was not eluted. Twenty-seven per cent of estrone was eluted by toluene–chloroform; a second band of different absorption spectrum was brought down by 3% ethanol in chloroform.

These results indicated that silicic acid containing little or no free water either irreversibly adsorbed sterols or altered their structure, as judged by the shape of their spectral absorption graphs in acid solution. Silicic acid containing 8% or more of free water was therefore used for subsequent recovery tests on 100 μgm. amounts of the available pure sterols.

Estrone recoveries were compared on a series of columns containing 1.5 gm. of silicic acid of different free-water contents. The results were as follows: 100%, 94%, and 35% recovered from silicic acid of 8%, 17%, and 32% respectively, water content. To test whether the decreased recovery resulted from the formation of a partition column with increasing water content, the water-insoluble neutral sterols were recovered from columns of the same free-water contents. DHA showed no change in recovery over this range. Androsterone recovery was 75%, and isoandrosterone recovery was 70% from columns containing 8% of free water. Isoandrosterone recovery was increased to 98% using a column containing 17% of free water. The volume of forerun and the volume containing the eluted steroid decreased with increasing free-water content. This indicated that the degree of adsorption on the solid phase was decreased.

The ability of the silicic acid adsorbent to separate a mixture of sterols was then examined. Androsterone differs from isoandrosterone only in the spacial configuration of the $C_3$—OH group. DHA differs from isoandrosterone only by a double bond at the $C_5$ position. The partial chromatographic separation of a mixture of these three on silicic acid containing 8% of water is illustrated in Fig. 2 by a graphical breakdown of the absorption curves. Toluene was used as the solvent and as the developer. Changes in free water, developer, and origin of sample made little change in the degree of separation; no complete isolation of these three from each other was attained.

A number of sterols possessing greater structural differences were then tested, using the same batch of silicic acid. A complete chromatographic separation from a mixture of cholesterol, estrone, dehydroisoandrosterone, pregnanediol, and estriol is shown in Fig. 3, together with an absorption curve of one fraction of each substance. The first three were separated by toluene developer; chloroform was required to elute the pregnanediol; and 30% alcohol in chloroform to elute the estriol.

Further experiments were carried out on the elution of estriol from 1.5 gm. columns containing 17% of free water. Six per cent alcohol in chloroform eluted the estriol in 80% yield without excessive tailing. No change in forerun of the alcohol solution was noted when the alcohol developer was preceded by pure chloroform. We concluded that the estriol was eluted by displacement.

These experiments indicated that the degree of adsorption of the sterols onto the solid phase increased with the number of sterol hydroxyl groups. As a further test, a mixture of the estrogen series (estrone, α-estradiol, and

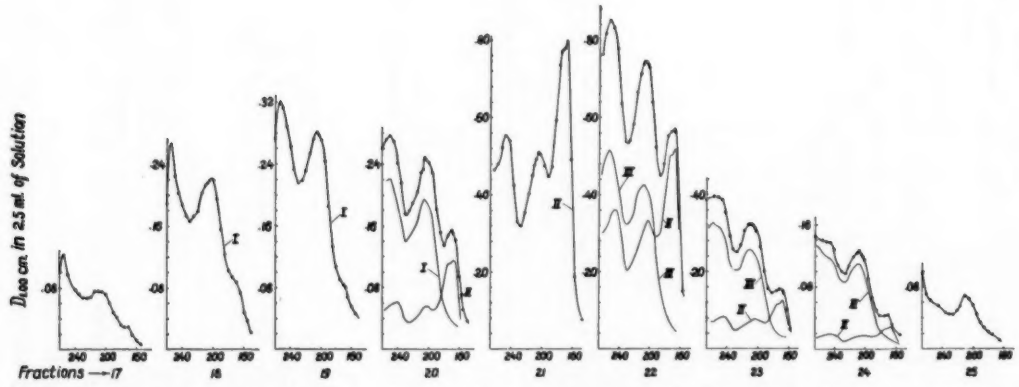


FIG. 2. Partial chromatographic separation of a mixture of: I, androsterone; II, dehydroisoandrosterone; III, isoandrosterone: absorption curves in sulphuric acid – methanol solution. Adsorbent: silicic acid containing 8% free water. Eluent: 1 : 1 : : toluene: chloroform by volume. (Abscissa: wave number $\times 10^{-3}$; ordinate: optical density of 1.00 cm.)

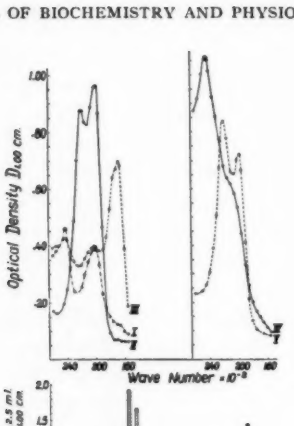


FIG. 3. Lower section: an example, in the sequence shown, of the complete chromatographic separation of a mixture of: I, cholesterol; II, estrone; III, dehydroisoandrosterone; IV, pregnanediol; V, estriol. Adsorbent: silicic acid containing 8% of free water. Eluent: 1 : 1 : : toluene: chloroform by volume for I, II, and III; chloroform for IV; 1 : 3 : : methanol: chloroform by volume for V. Recoveries: I, 86%; II, 100%; III, 71%; IV, 86%; V, 81%. Upper section: a characteristic absorption spectrum of each eluted fraction: I, fraction 11; II, fraction 17; III, fraction 21; IV, fraction 31; V, fraction 33.

estriol in 1.0 ml. of chloroform) were introduced into a column of 1.5 gm. of silicic acid containing 17% of free water. The results are shown in Table I.

TABLE I

| Eluents | Total volume of forerun (ml.) | Estrogen | Volume containing estrogen (ml.) | % recovery |
|---|---|---|---|---|
| (*a*) 48 ml. | 24 | Estrone | 6 | 91 |
| (*b*) 12 ml. | 44 | α-estradiol | 8 | 68 |
| (*c*) 24 ml. | 66 | Estriol | 5 | 78 |

*The eluents used in succession were: (a) 48 ml. of a mixture of toluene and chloroform in equal volumes; (b) 12 ml. of 2% ethanol in chloroform; (c) 24 ml. of 6% ethanol in chloroform.*

*Separation of Urine Components*

Urine was hydrolyzed and the free steroids extracted with organic solvents. Hydrolysis procedures have been discussed extensively in the literature (7). Hot acid hydrolysis releases the greatest number of substances from their water-soluble conjugates, and in addition produces a number of artifacts. We selected this procedure to test the ability of the combined spectrometric and chromatographic methods to characterize the components of a complex

mixture. To one volume of urine was added 1/10th volume of concentrated hydrochloric acid and one-half volume of toluene. The mixture was boiled for 15 min. under reflux, then cooled. The toluene layer was washed with 0.1 *N* sodium hydroxide and with water, then gently boiled to remove the water, the boiling being continued until the toluene volume was reduced to 5–10 ml. Aliquots of the toluene solution were then used for the chromatographic tests.

To date, only the neutral urine components extracted from the aqueous layer by toluene have been studied. Of these compounds cholesterol, androsterone, dehydroisoandrosterone, and possibly isoandrosterone (this may be etiocholanolone) have been tentatively identified (*a*) by comparison with the chromatographic behavior of pure samples; (*b*) by addition of these compounds in excess to one-half a urine sample and the carrying out of duplicate analyses; and (*c*) by their absorption in the sulphuric acid – methanol reagent and the Pincus reagent (Ref. 7, p. 144). The remainder have been arbitrarily labelled in the order of their elution. The sequence is: "$R_1$", "A", "B", cholesterol, "$C_1$", "$C_2$", pigment band, "E", androsterone, dehydroisoandrosterone, isoandrosterone, "K", "L", "M", and "N". These substances show clearly resolved and easily recognized absorption maxima in the acid–alcohol reagent. Substances having a low general absorption which occur within the sequence are termed residual substances, "R".

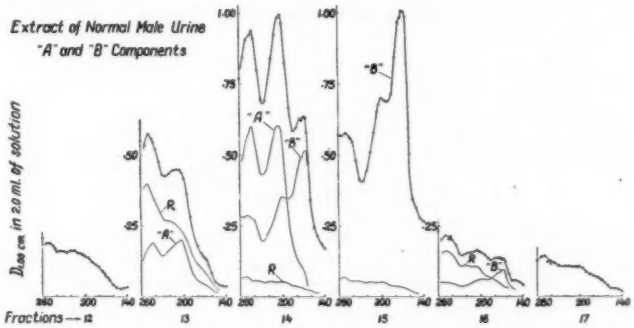


FIG. 4. Chromatographic separation of "$R_1$", "A", and" B" from toluene extract acid-hydrolyzed urine; absorption spectrum of each fraction in sulphuric acid – methanol reagent. Adsorbent: silicic acid containing 8% of free water. Eluent: 1 : 1 : : toluene: chloroform by volume. (Abscissa: wave number $\times 10^{-2}$; ordinate: optical density of 1.00 cm.)

Fig. 4 is an example of the elution sequence of compounds "$R_1$", "A", and "B" in 5.0 ml. fractions of eluate. The complete absorption curve of the material in each fraction, after treatment with the acid–alcohol color reagent, is plotted; this sequence illustrates the method of graphical analysis of a mixture based on the selection of an absorption curve for each component in the pure state. The fractions following, which contain cholesterol and "C" substances, are similarly shown in Fig. 5.

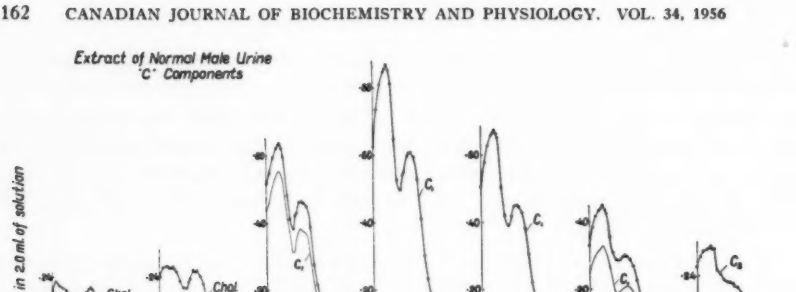


FIG. 5. Chromatographic separation of cholesterol, "$C_1$" and "$C_2$" from toluene extract of acid-hydrolyzed urine; absorption spectrum of each fraction in sulphuric acid – methanol reagent. Adsorbent: silicic acid containing 8% of free water. Eluent: 1 : 1 : : toluene: chloroform by volume. (Abscissa: wave number $\times 10^{-2}$; ordinate: optical density of 1.00 cm.)

The substances eluted in fifty-four 10.0 ml. fractions from the overnight urine of a normal male are shown in Fig. 6 where the androsterone and dehydroisoandrosterone were well separated from the purple pigment band. A series from a slightly wetter sample of adsorbent is shown in Fig. 7. The

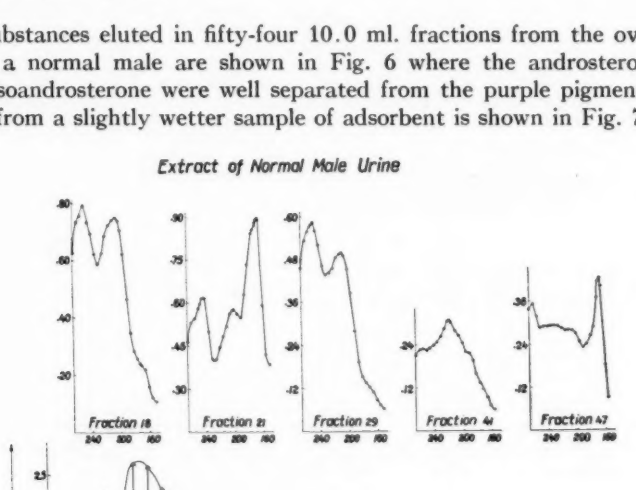


FIG. 6. Chromatographic separation of androsterone to "L" from toluene extract of acid-hydrolyzed urine. Adsorbent: silicic acid containing 8% of free water. Eluent: 1 : 1 : : toluene: chloroform by volume.

substances are distinguished from one another both by the shapes of the absorption curves and by the chromatographically separated bands that are apparent in the sequence of fractions. From each band an absorption curve typical of that substance has been selected on the assumption that the impurities are low when the same shape persists through several fractions. These curves have been used to analyze graphically the fractions at the overlap of the bands.

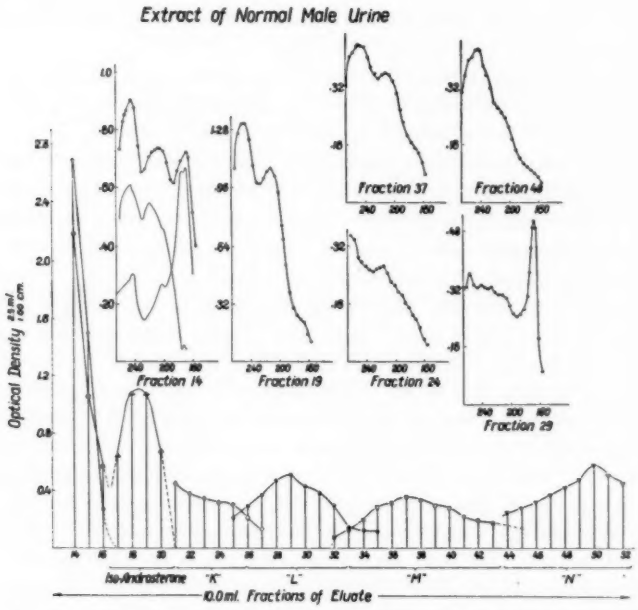


FIG. 7. Chromatographic separation of androsterone to "N" from toluene extract of acid-hydrolyzed urine. Adsorbent: silicic acid. Eluent: 1 : 1 : : toluene: chloroform by volume.

*Comparisons of Hot and Cold Hydrolyses*

Hot acid hydrolysis of urine produces two artifacts of dehydroisoandrosterone, the $C_3$-chloro derivative and the $C_3$ conjugated unsaturated derivative. To determine where these compounds occurred in the chromatogram relative to the DHA, a comparison of the ketonic fractions from hot- and cold-hydrolyzed urine was made.

Pooled urine from a male was divided into two lots, each of 1 liter. We hydrolyzed one lot by boiling it with 100 ml. of hydrochloric acid, overlaid with 500 ml. of toluene. The boiling time was 15 min., but a prior 15 min. heating was required to bring it to boiling point. The second lot was acidified with 100 ml. of hydrochloric acid and continuously extracted with ether for 48 hr. The ether was evaporated and the residue taken up in toluene.

The separation of the ketosteroid fraction was carried out as follows:

The toluene extract of the acid-hydrolyzed sample of urine was reduced to 20 ml. by distillation, then evaporated to dryness in a test tube at 55° C. by a stream of nitrogen. The residue was dissolved in 5.0 ml. of anhydrous methanol, to which was added 250 mgm. of Girard's reagent T and 0.5 ml. of glacial acetic acid. After 24 hr. at room temperature the tube was cooled in ice water while 1.40 ml. of 6 *N* sodium hydroxide was added, to "nine-tenths" neutralize the glacial acetic. Twenty milliliters of ice water was then added to the test tube and the non-ketosteroids were removed by extracting the mixture three times with 10 ml. of toluene.

The aqueous phase, containing the ketosteroids, was acidified to pH of 1.0 with sulphuric acid, and after 20 hr. at room temperature it was extracted three times with 10 ml. of toluene to remove the ketosteroids.

The toluene solutions were washed with 3 *N* sodium hydroxide and water. They were boiled to remove the water and reduce the volumes of toluene to 5.0 ml.; chromatographic analyses were carried out on 1.0 ml. aliquots.

The chromatographic columns were made up of 1.5 gm. silicic acid in 6 mm. diameter tubes; two values of free water were tested. A mixture of equal volumes of toluene and chloroform was used as the eluent.

In the presence of 17% free water, the extract from the cold hydrolysis produced a single band between 24 and 29 ml. of eluate as shown in the upper series of graphs in Fig. 8. This band was made up of an unsaturated ketosteroid, having an absorption maximum at 16,800 $cm.^{-1}$ and identified as DHA by analogy with a pure sample, and a second component partially superimposed. The curve shape of the latter indicates a saturated ketosteroid.

In the presence of 17% free water, the extract from the hot hydrolysis produced the elution pattern shown in the lower half of Fig. 8. The unsaturated ketone "B" was eluted in fractions 8 to 12, together with a substance having a single sharp maximum at 20,200 $cm.^{-1}$. A second band extended from fractions 25–36 of which fractions 30–36 were of practically the same absorption shape and intensity. The position of this second band, together with the absorption spectra, indicated it to be the same saturated ketosteroid as was produced in the cold hydrolysis, but in much greater quantity.

The occurrence of the unsaturated ketosteroids "B" and DHA were evidently related to the method of hydrolysis. The urine sample previously hydrolyzed cold was then resubjected to hot hydrolysis, Girard T extraction, and chromatography. A further small amount of "B" only was obtained. We concluded that "B" was formed from DHA by hot acid hydrolysis and was probably the 3-chloro derivative or the conjugated unsaturated compound. Because DHA is not affected by hot 10% hydrochloric acid, the change must occur at the instant the conjugate link is broken.

To determine whether the presence of a greater amount of free water would permit the elution of additional steroids, aliquots of the extracts from both the cold and hot hydrolyses were chromatographed on silicic acid

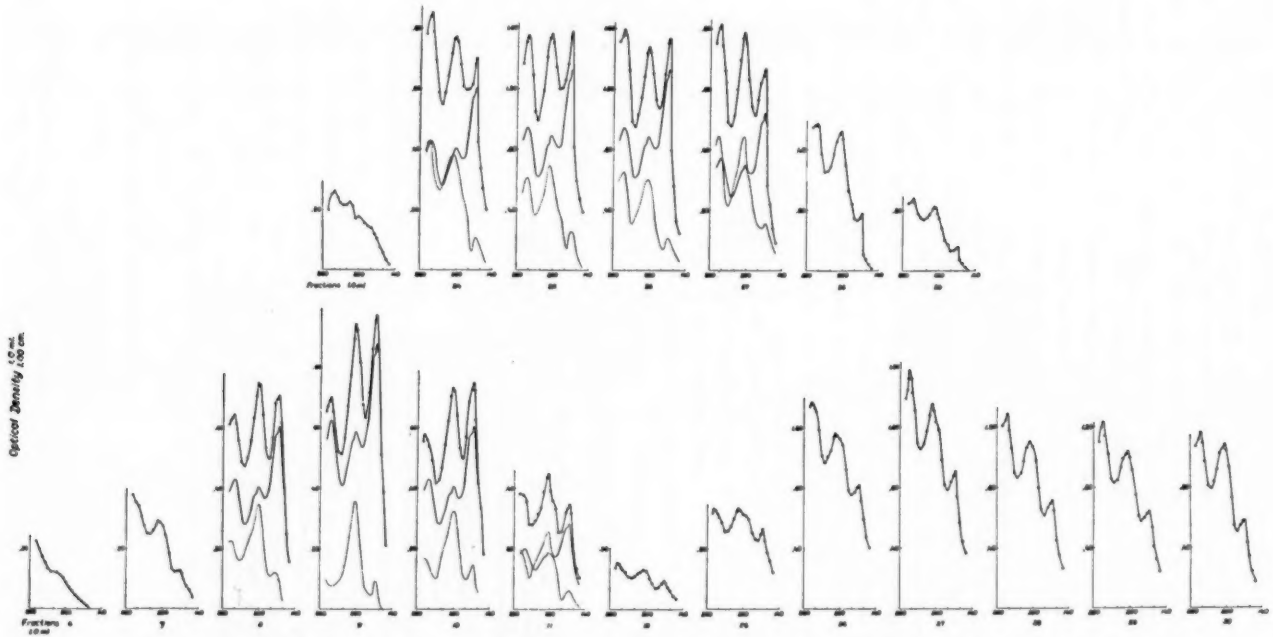

FIG. 8. Chromatographic elution of ketosteroid fraction from acid-hydrolyzed urine. Adsorbent: silicic acid containing 17% of free water. Upper curves: urine hydrolyzed 48 hr. at room temperature. Lower curves: urine hydrolyzed 30 min. by boiling.

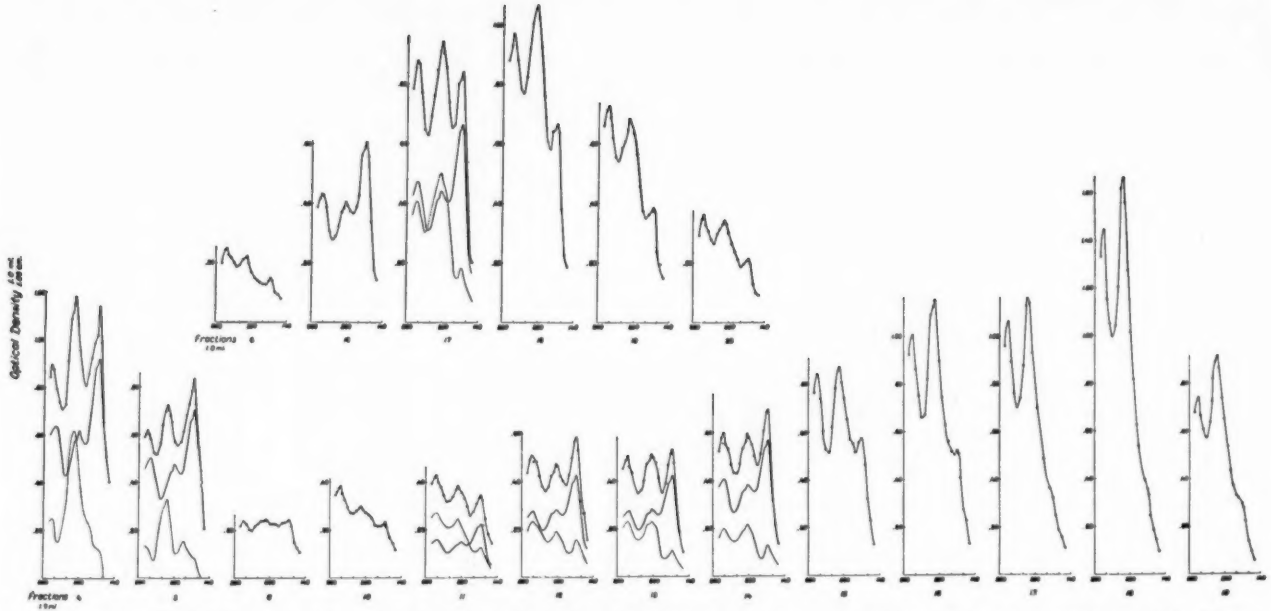

FIG. 9. Chromatographic elution of ketosteroid fraction from acid-hydrolyzed urine. Adsorbent: silicic acid containing 32% of free water. Upper curves: urine hydrolyzed 48 hr. at room temperature. Lower curves: urine hydrolyzed 30 min. by boiling.

containing 32% of free water, Fig. 9. This did not materially alter the elution pattern of the extract from the cold hydrolysis; compare the upper curves of Fig. 9 with Fig. 8. The front of the DHA band was advanced to fraction 16, from fraction 24, but the same materials are present. Using the wetter silicic acid with the extract from the hot hydrolysis, however, produced two eluted bands, the fractions of which showed the absorption spectra of unsaturated ketones. Compare the lower curves of Fig. 9 with those of Fig. 8. The "B" band, accompanied by the component having a maximum at 20,200 cm.$^{-1}$, is present as before. In addition a band extends over fractions 11 to 14. The two possible products of hot acid hydrolysis, the 3-chloro and the 3-unsaturated derivatives, may be represented here.

The non-ketonic component following cold hydrolysis contained little material. A spot check of the fractions, obtained by chromatography of the non-ketonic material following hot acid hydrolysis, produced many of the absorption curves shown in Figs. 5 and 6. These may include substances more difficult to hydrolyze, as well as possible artifacts. At this point, a study of the results in Figs. 4, 6, 8, and 9 leads us to conclude that the "A" curve of Fig. 4 does not represent a single substance. It is probably composed of the "$R_1$" curve in that figure and of a component, shown in fractions 8 to 11 in the lower part of Fig. 8, which has a single sharp maximum at 20,200 cm.$^{-1}$. Furthermore, fraction 47 of Fig. 6 may be the same substance as appears in fractions 11 to 14 in the lower part of Fig. 9; a high degree of retention by the drier column in the former case could cause the difference in absorption.

A sample of the ketonic fraction following hot hydrolysis was chromatographed on silicic acid containing no free water. The elution pattern obtained was similar to fractions 7 to 12 shown in the lower part of Fig. 8; the band, however, was spread over fractions 19 to 32 and the resolution was better. We concluded that the "B" component is adsorbed by the silicic acid to a very small extent, and that no hydroxy steroids could be present in this group.

A good example of the method of analyzing the overlap of two bands, using the characteristic absorption of sterols in the acid–alcohol reagent, is shown by the upper curves of Fig. 9. Fraction 17 is composed of a mixture of fractions 16 and 18. This is shown by measuring $D$ on the three graphs at the same wave number $\nu_1$ and again at a second wave number $\nu_2$. Maxima or minima are the most dependable points. The equations were written: $P_1D^{16}_{\nu_1} + P_2D^{18}_{\nu_1} = D^{17}_{\nu_1}$ and $P_1D^{16}_{\nu_2} + P_2D^{18}_{\nu_2} = D^{17}_{\nu_2}$, where $D^x_\nu$ refers to density of fraction $x$ at wave number $\nu$ and $P_1$ is proportion of fraction 16, $P_2$ is proportion of fraction 18 that together make up the composite fraction 17.

The equations were solved for $P_1$ and $P_2$.

## Conclusions

Silicic acid reversibly adsorbs water vapor; its properties as an adsordent for sterols can be modified by changing this free-water content. Up to

17% of free water, adsorption at the solid surface appears to predominate. At 32% of free water, partition effects appear with water-soluble sterols. In the absence of free water the sterols are chemically altered.

The chromatographic adsorption of sterols by silicic acid is related to the presence of the hydroxyl groups in the steroid molecule. In the presence of free water, mono and dihydroxy sterols equilibrate themselves between surface and solvent phases; the dihydroxy sterol appeared to be more firmly bound to the surface phase. The distribution is progressively displaced towards the solvents petroleum ether, toluene, chloroform, in that order, and is also displaced to the solvent phase by increasing the amount of free water. The trihydroxy sterol appeared to be completely adsorbed on the solid phase and could only be recovered from the surface by displacement with ethanol.

Changes affecting the hydroxyl group are therefore readily detected; the products are well separated from the parent sterol. Some steroid products of hot acid hydrolysis are not retained by dry silicic acid, owing to loss of the hydroxyl group.

The acid–alcohol color reaction detected a number of sterols in urine extracts. In cases where the eluted bands were not resolved, the extent of admixture could be determined.

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# ENZYMATIC PROPERTIES OF BOVINE PLASMIN PREPARATIONS; EVIDENCE FOR SIMILARITY TO BUT NON-IDENTITY WITH TRYPSIN[1]

By Edward Ronwin[2]

## Abstract

The enzymatic properties of both choloroform-only activated and trypsin–chloroform activated bovine fibrinolysin (plasmin) have been studied and compared with trypsin. These studies accentuate the remarkable similarity between plasmin and trypsin but definitely establish their distinct individuality and the validity of the procedure using trypsin to activate plasminogen to plasmin.

## Introduction

This investigation was undertaken to elucidate the properties of the trypsin-activated bovine fibrinolytic enzyme (plasmin) and to establish its non-identity with trypsin. Utilizing the simple substrate, $N^{\alpha}$-*p*-toluenesulphonyl-L-arginine methyl ester (12), various facets of the activity of the purest bovine plasmin yet prepared were studied. However, the preparation is admittedly still far from uncontaminated. The results emphasize the amazing similarity but non-identity of plasmin and trypsin.

## Materials and Methods

*Measurement of Enzyme Action*

The measurement of the hydrolytic activity was conducted by a continuous titration procedure in the presence of a nitrogen gas flush of the reaction mixture and under the following general set of conditions (which, of course, were altered when the effect of any particular condition was under examination): $S_0$, 0.01 *M*; temp., 38° C.; pH, 8.05 ± 0.02; buffer, 0.015 *M* veronal. In most cases, sufficient substrate was dissolved in 1.5 ml. of buffer (maintained at bath temperature) and 1 ml. of this solution was taken for the ES reaction mixture. The remaining portion served as the substrate control. An amount of enzyme (weighed, in dry condition, on an analytical balance capable of measurements to $10^{-6}$ gm.) was similarly dissolved in 1.5 ml. of buffer. When a milliliter aliquot of enzyme solution was added to the milliliter of substrate solution, the desired initial concentrations of all components was realized. The time of addition of the enzyme to the substrate was taken as the zero point of the reaction. At this time a small quantity of concentrated base (usually 0.01 ml. of 0.2 *N* base) was added to the reaction mixture with shaking and the time for neutralization of the added base was recorded. The neutralization point was read on the galvanometer of a Beckman model G pH meter. As soon as neutralization occurred this process was repeated. Usually a dozen points were read for any one reaction run.

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*Enzyme Preparation*

The preparation of plasmin 1E (the 1E is merely a designation of one of the preparations in this work) was performed in precisely the same manner as described by Jackson and Mertz (3). Preparation 1E was chloroform–trypsin activated. The preparation labelled 1D was chloroform-*only* activated (3). The plasminogen preparation was obtained in essentially the same fashion as the plasmins minus the activation steps (3). Preparations 1D and plasminogen 11M54 were available in this laboratory through the work of Dr. H. D. Jackson prior to the start of this investigation. The trypsin used was a commercial preparation purchased from Armour and Co. which contains about 40% $MgSO_4$. It was found to have constant activity and used as such.

*Substrates and Inhibitors*

The following substrates were used: $N^{\alpha}$-*p*-toluenesulphonyl-L-arginine methyl ester . HCl (TAME); L-arginine methyl ester . dihydrochloride (AMED); $N^{\alpha}$-3,5-dinitrobenzoyl-L-arginine methyl ester . HCl (3,5-DNBAME); $N^{\alpha}$-*m*-nitrobenzoyl-L-arginine methyl ester . HCl (MNBAME); $N^{\alpha}$-benzoyl-L-arginine methyl ester . carbonate (converted to the hydrochloride prior to use) (BAME). TAME was a commercial product of the H & M Chem. Co., Ltd., Santa Monica, California. AMED was a product of Eastman Kodak. The other compounds were generous gifts in pure condition from Dr. A. K. Balls of this department.

The following compounds were employed in inhibition studies: taurine (EK product); AMED (see above); L-arginine . HCl (EK product); $N^{\alpha}$-*p*-toluenesulphonyl-L-arginine (H & M Chem. Co.); N-*p*-toluenesulphonylglycine (synthesized by the method of McChesney and Swann (5) ); *p*-chloromercuribenzoic acid (Sigma Chem. Co.); $N^{\alpha}$-*m*-nitrobenzoyl-L-arginine and $N^{\alpha}$-benzoyl-L-arginine (BA). The last three compounds were gifts from Dr. A. K. Balls. The inorganic salts were standard commercial analytical products.

## Results and Discussion

*pH Optima for Bovine Fibrinolysin (Plasmin 1E)*

Fig. 1 shows the pH optima for plasmin 1E in tris(hydroxymethyl)aminomethane, borate–borax, and veronal buffers, and in pH-adjusted water. The curve for the adjusted water situation fits very well with any curve which might be drawn to generalize the pH dependency of the enzyme in any of the buffers. This curve is very similar to that obtained for trypsin on the same substrate when part of the curve is determined in phosphate buffer and part in borate buffer (10). Consequently, the pH optimum behavior fails to demonstrate a difference in action between trypsin and plasmin (which is trypsin activated and thereby suspect to trypsin contamination). The curve does demonstrate that variations in buffer concentration from 0.00 to 0.05 *M* have relatively little effect on the pH optimum behavior of plasmin.

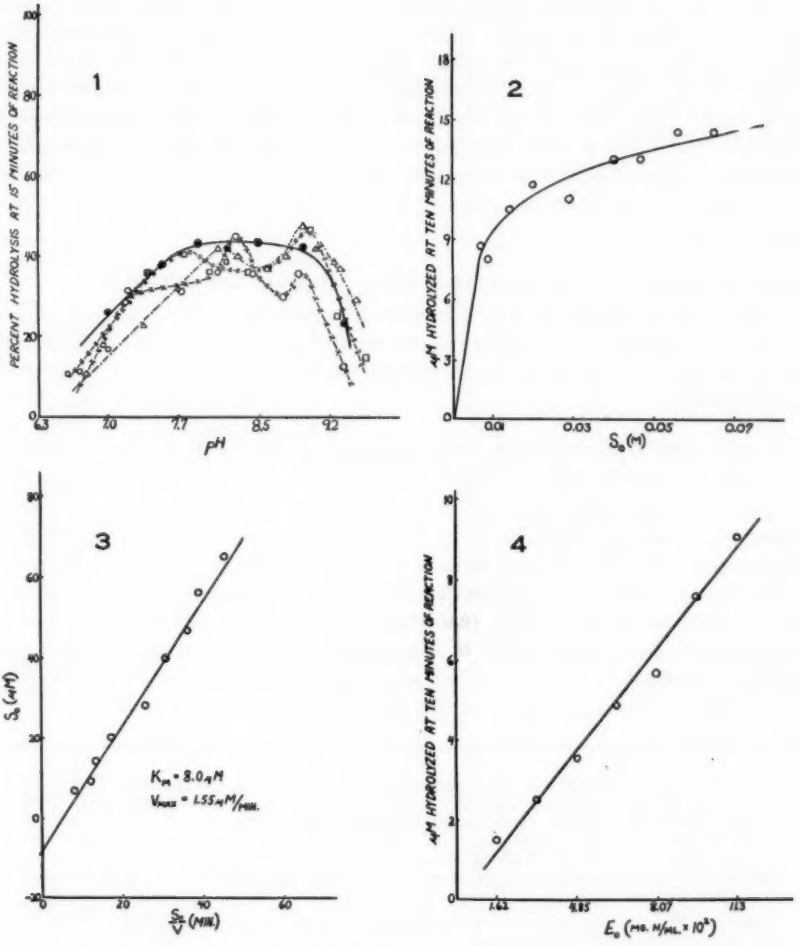


FIG. 1. The pH optimum for plasmin 1E.
$S_0$: 0.02 *M* TAME; temp.: 38° C.; $E_0$: 0.097 mgm. N/ml.
—x—x—○— 0.05 *M* Tris buffer. —||—||—□— 0.05 *M* borate–borax buffer. —·—·—△— 0.05 *M* veronal buffer. —●— pH adjusted water.

FIG. 2. The effect of initial substrate concentration on rate of plasmin 1E action. pH: 8.05; temp.: 38° C.; buffer: 0.05 *M* veronal; $E_0$: 0.097 mgm. N/ml.; TAME substrate.

FIG. 3. The Lineweaver–Burk plot for plasmin 1E. pH: 8.05; temp.: 38° C.; buffer: 0.05 *M* veronal; $E_0$: 0.097 mgm. N/ml.; TAME substrate.

FIG. 4. The rate of hydrolysis as a function of initial enzyme (plasmin 1E) concentration. $S_0$: 0.00933 *M* TAME; pH: 8.05; temp.: 38° C.; buffer: 0.015 *M* veronal.

*Determination of $K_m$, $V_{max}$, and $k_3$ for Plasmin 1E*

The effect of variations in the initial substrate concentration upon plasmin activity is pictured in Fig. 2. When subjected to Michaelis–Menten kinetic concepts, the data yield the curve shown in Fig. 3. From this curve the values of $K_m$, $V_{max}$, and $k_3$ were found to be, respectively: 8.0 $\mu$M.; 1.55 $\mu$M./min.; 16.0 $\mu$M.-ml./min.-mgm.N. These values have to be taken with caution, since the enzyme concentration value used in their calculation is subject to considerable error when viewed in light of other data to be presented here, which show that the plasmin preparation is still contaminated with plasminogen.

*Rate of Hydrolysis as a Function of Initial Enzyme Concentration*

Variations in initial enzyme concentration and their effect on the rate of TAME hydrolysis by plasmin 1E are plotted in Fig. 4. The data clearly show that the rate of hydrolysis is proportional to enzyme concentration over a wide range.

*Demonstration of Plasminogen Contamination in the Plasmin 1E Preparation and Determination of its Rate of Activation*

Fig. 5 demonstrates that the combined action (at equal *activity concentrations*) of trypsin and plasmin 1E on TAME yields a rate of hydrolysis which is higher than the sum of the activities of both enzymes at the same concentrations acting individually. Since the plasmin 1E and plasminogen are prepared in an identical manner, it is reasonable to conclude from the results that plasminogen is a contaminant of the plasmin 1E preparation and that the additional activity observed in the combined action of plasmin 1E and trypsin on TAME is due to activation of the plasminogen contaminant to plasmin by

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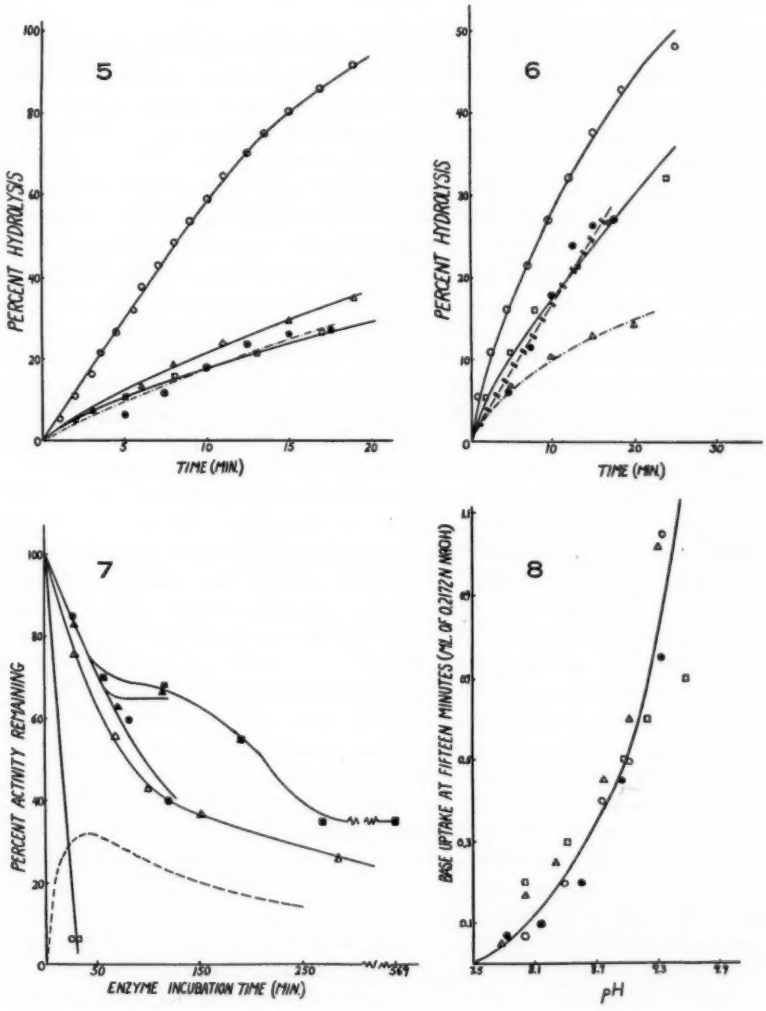
FIG. 5. The demonstration of plasminogen contamination in the plasmin 1E preparation. $S_0$: 0.01 *M* TAME; temp.: 38° C.; pH: 8.05; buffer: 0.015 *M* veronal; plasmin 1E, $E_0$: 0.049 mgm. N/ml.; trypsin, $E_0$: 0.000088 mgm. N/ml.
○—Plasmin 1E plus trypsin. □—Trypsin alone. △—Plasmin 1E alone. ●—Activity due to activation of plasminogen contaminant in the plasmin 1E preparation. (Difference in activity of plasmin 1E plus trypsin combined minus the sum of the activity of the two enzymes alone.)

FIG. 6. The trypsin activation of plasminogen. $S_0$: 0.01 *M* TAME; temp.: 38° C.; pH: 8.05; buffer: 0.015 *M* veronal.
○—Trypsin (0.000088 mgm. N/ml.) plus plasminogen 11M54 (0.104 mgm. N/ml.). □—Trypsin only (0.000088 mgm. N/ml.). △—Constructed from difference in action of trypsin plus plasminogen minus action of trypsin alone. ●—Constructed from computed plasminogen activation in situation described in FIG. 5.

FIG. 7. The stability curves for plasmin 1E, plasmin 1D, and trypsin. Enzymes incubated at pH 8.0, 38° C. in 0.015 *M* veronal buffer. Activities determined on 0.01 *M* TAME, 38° C., pH 8.05 in 0.015 *M* veronal buffer. ○—Trypsin, incubated at 0.00072 mgm. N/ml. □—Trypsin, incubated at 0.0396 mgm. N/ml. △—Trypsin plus plasmin 1E, trypsin incubated at 0.00072 mgm. N/ml. and plasmin 1E at 0.194 mgm. N/ml. ●—Plasmin 1E, incubated at 0.194 mgm. N/ml. ■—Plasmin 1D, incubated at 0.190 mgm. N/ml. ▲—Plasmin 1D, incubated at 0.190 mgm. N/ml.

FIG. 8. The spontaneous hydrolysis of TAME. $S_0$: 0.02 *M*; temp.: 38° C.
○—0.05 *M* Tris buffer. □—0.05 *M* borate–borax buffer. △—0.05 *M* veronal buffer. ●—pH adjusted water.

trypsin. The formation of plasmin from an individual plasminogen preparation of trypsin was also studied; the results are graphically presented in Fig. 6. The difference in rate of hydrolysis observed when trypsin and plasminogen are both present in the reaction mixture, as compared to the rate observed when trypsin alone is present (plasminogen does not possess any hydrolytic activity under the test conditions) can only be attributed to the conversion of plasminogen to plasmin by trypsin. The function represented by the solid



circles and the cross-tied line was constructed from the rate of plasminogen to plasmin conversion observed in Fig. 5 wherein the trypsin concentration was equivalent to that present in Fig. 6. The higher rate of activity may be due to the fact that the plasminogen (Fig. 6) was not previously treated with chloroform, whereas the plasmin 1E preparation supplying plasminogen for activation by trypsin (Fig. 5) has been chloroform treated. It is known that the chloroform treated material can be activated to a greater degree than the non-chloroform treated plasminogen by equal quantities of trypsin (3).

*Stability Curves for Plasmin 1E, Plasmin 1D, and Trypsin*

A study of the stability of these three enzymes in 0.015 *M* veronal buffer solutions at pH 8.0 and 38° was undertaken. Besides serving as an indicator of the permissible time period during which the enzymes could be so treated, before testing, without significant loss of activity, these studies, it was hoped, would demonstrate a clear-cut difference between the behavior of the plasmins and trypsin. As can be seen from Fig. 7, trypsin, both in dilute and concentrated solutions, rapidly decomposes. On the other hand, both of the plasmins have considerably more stability under the incubation conditions. It appears safe to handle plasmin up to 20 min. after the preparation of the solution without significant loss of activity. A curve was run to determine the stability of a solution of plasmin 1E plus trypsin under these conditions. The resultant curve is given by the open triangles, Fig. 7. This decomposition curve is somewhat at variance (represents a lower rate of decomposition) with that expected from a trypsin – plasmin 1E solution assuming that trypsin and plasmin 1E break down at their respective rates when incubated alone in a similar fashion. The variation can be explained by the fact that the trypsin is rapidly converting the plasminogen of the plasmin 1E preparation to plasmin, thereby adding a component which breaks down much more slowly than trypsin alone, thus slowing down the breakdown rate of the combined trypsin – plasmin 1E solution. The asymptotic approach of this curve toward a zero value can be ascribed to the continually decreasing plasminogen conversion to plasmin while the plasmin originally present, plus the added plasmin, slowly decomposes. The dashed line on the graph has no quantitative significance but was included to give the reader an idea of the rate of plasmin formation from plasminogen which apparently takes place.

Plasmin 1D over a period of 60 min. for both runs shown in Fig. 7 exactly coincided in breakdown rate with that for plasmin 1E alone. The curve for plasmin 1D appears to level off after 60 min. for a period of approximately 100 min. and then begins to fall and approach a zero point asymptotically. This variation in behavior from the plasmin 1E and plasmin 1E – trypsin breakdown curves might find explanation in the consideration that the plasmin content on a nitrogen activity basis for plasmin 1D is much lower than for plasmin 1E and that the additional nitrogen content of the plasmin 1D preparation is mainly plasminogen. The resulting balance of factors, that is, the conversion of plasminogen to plasmin, and plasmin breakdown in 1D, is somewhat altered as compared to the balance of factors in the other two cases.

*Spontaneous Hydrolysis of TAME*

Fig. 8 shows the spontaneous hydrolysis of TAME determined in three different buffers and in pH-adjusted water. While there are some wide variations from the curve, it can be seen that a definite trend is discernible. Since the value for the spontaneous hydrolysis of this substrate in the area of pH 8 is virtually insignificant, most operations were carried out at this pH, which corresponds closely to the maximum pH optimum for plasmin 1E. It is obvious from the curve that the higher the pH, the greater the rate of spontaneous hydrolysis.

*Comparative Specificities of Plasmin 1E, Plasmin 1D, and Trypsin*

The action of these three enzymes on various substrates was determined under similar reaction conditions and the results are presented in Table I. As presented, valid comparisons can only be made within a column. While relative comparisons can be made across columns, these comparisons have no quantitative significance as a result of the mode in which the data are presented. The most glaring difference in observed activities is that trypsin has a much greater rate of comparative activity on TAME as compared to the other substrates than either of the other two enzymes. The differences in

TABLE I

COMPARATIVE SPECIFICITIES OF PLASMIN 1E, PLASMIN 1D, AND TRYPSIN

Common conditions.—temp.: 38° C.; pH: 8.05 ± 0.02; buffer: 0.015 *M* veronal.
Unless otherwise noted, the enzyme concentrations were: plasmin 1E, $E_0$: 0.097 mgm. N/ml.; plasmin 1D, $E_0$: 0.190 mgm. N/ml.; trypsin, $E_0$: 0.00018 mgm. N/ml.
Trypsin and plasmin 1E are at equal activity concentrations using TAME as substrate.

| Substrate ($S_0$: 0.01 *M*) | Plasmin 1E | | Plasmin 1D | | Trypsin | |
|---|---|---|---|---|---|---|
| | % Hyd.[a] | Ratio: TAME/other compound | % Hyd.[a] | Ratio: TAME/other compound | % Hyd.[b] | Ratio: TAME/other compound |
| TAME[c] | 59.0 | | 30.7 | | 32.2 | |
| AMED | 0.0[d] | | | | 0.04[e,f] | |
| 3,5-DNBAME | 13.4 | 4.4 | 11.5 | 2.7 | 2.6[f] | 12.4 |
| MNBAME | 7.3 | 8.1 | 15.9 | 1.9 | 1.6[f] | 20.1 |
| BAME | 8.8 | 6.7 | 21.1 | 1.5 | 2.9[f,g] | 11.1 |
| BAME plus 0.01 *M* BA | | | | | 3.2[f,h] | 10.0 |

[a] *Rate at 15 min. of reaction.*
[b] *Rate at 6 min. of reaction.*
[c] *For abbreviations see Experimental Section.*
[d] *After one hour.*
[e] *Reaction failed to proceed beyond 11% hydrolysis.*
[f] *Value obtained after adjustment to E concentration used for TAME.*
[g] *From run made at an enzyme concentration of 0.0015 mgm. N/ml.; a value of 2.6 was obtained at an enzyme concentration of 0.0044 mgm. N/ml. This permits the conclusion that a proportionality exists between enzyme concentration and rate of hydrolysis over this range.*
[h] *From run made at an enzyme concentration of 0.0022 mgm. N/ml. (within the range of proportionality between rate of hydrolysis and enzyme concentration); a value of 3.2 was obtained from extrapolation of results from a run at an enzyme concentration of 0.00088 mgm. N/ml.*

TAME/*other compound* ratios observed for plasmin 1E and plasmin 1D may be due to the difference in plasminogen : plasmin ratios of these enzyme preparations. Attention is drawn to the fact that benzoyl-L-arginine is not inhibitory toward trypsin when BAME is the substrate. The significance of this will be discussed further when the effect of various organic agents upon plasmin 1E and trypsin is discussed.

*Acid–Heat Treatment Studies*

It was previously shown by Kunitz and Northrop (4) that trypsin can be treated in 0.01 *M* HCl solutions at various temperatures below 100° C. and for various time periods without loss of significant hydrolytic activity. Since the specificities of plasmin 1E, plasmin 1D, thrombin, and trypsin are similar, a study was made to determine what effect acid–heat treatment would have on their respective hydrolytic abilities. In addition, the capacity for plasminogen to be converted to plasmin after acid–heat treatment was also observed. The results are presented in Table II. It will be noted that, after five minutes in HCl solution at pH 2.2 and 60°–70° C. followed by rapid cooling, adjustment to pH 8.05, and testing of activity, plasmin 1E, plasmin 1D, and trypsin incubated at dilute and concentrated solutions maintain at least 75% or more of the activity possessed before treatment. It is interesting that the more concentrated trypsin solution appeared to be better protected under these conditions than the dilute trypsin solution. On the other hand,

TABLE II

COMPARATIVE STUDY OF PLASMIN 1E, PLASMIN 1D, TRYPSIN, PLASMINOGEN,[a] AND THROMBIN ACTIVITIES AFTER ACID–HEAT TREATMENT

Common conditions.—$S_0$: 0.01 *M* TAME; temp.: 38° C.; pH: 8.05 ± 0.02; buffer: 0.015 *M* veronal.

$E_0$, unless otherwise noted.—plasmin 1E: 0.097 mgm. N/ml.; plasmin 1D: 0.190 mgm. N/ml.; trypsin: 0.00018 mgm. N/ml.[b]; plasminogen 11M54: 0.104 mgm. N/ml.; thrombin: 5 NIH units/ml.

Experimental procedure.—The enzyme was incubated for five minutes in 0.01 *M* HCl (pH 2.2) at 60°–70° C. The solution was then rapidly cooled in an ice–salt bath and the pH quickly adjusted to 8.05 ± 0.02. The activity was then followed.

| Enzyme | Incubation temperature and incubation enzyme conc. | Relative activity[c] |
|---|---|---|
| Plasmin 1E | 63° C.; 0.194 mgm. N/ml. | 0.86 |
| Plasmin 1D | 63° C.; 0.380 mgm. N/ml. | 0.76 |
| Trypsin | 63° C.; 0.176 mgm. N/ml. | 0.91 |
| Trypsin | 60° C.; 0.00072 mgm. N/ml. | 0.75 |
| Thrombin | 65° C.; 10 NIH units/ml. | 0.18 |
| Plasminogen 11M54[d] plus trypsin[f] | 69° C.; 0.208 mgm. N/ml.[e] | 0.90 |

[a] *In this case, ability to be activated was studied.*
[b] *Concentration yields an activity equal to that of plasmin 1E.*
[c] *Activity of non-treated enzyme (% hydrolysis at 15 min. of reaction) taken as 1.00.*
[d] *Shown to have no hydrolytic activity alone whether or not it is acid–heat treated.*
[e] *Plasminogen only incubated; trypsin added immediately after the plasminogen to substrate test solution.*
[f] $E_0$: *0.000088 mgm. N/ml.*

thrombin action on TAME is virtually destroyed by the acid–heat treatment to which it was subjected. This is in accord with the results of thrombin action on fibrinogen under nearly similar conditions observed by Seegers (11). It is significant that plasminogen virtually maintained its ability to be converted to plasmin by trypsin despite the acid–heat treatment. The similarity of the plasmin and trypsin behavior after acid–heat treatment bespeaks similar active center geometry and structure. It is significant that another blood protein, albumin, is also known to possess this acid–heat resistance. These studies may prove, of some value in future plasmin purification efforts.

*Study of the Inhibition of Plasmin 1E by Various Organic Agents; Some Comparisons with Trypsin*

Table III is a compendium of the observations made on the inhibitory qualities or lack of the same by various organic agents which possessed some structural similarity to TAME or the other substrates listed in Table I. Surprisingly, taurine proved to be somewhat activating toward plasmin 1E without indicating this activity toward trypsin. L-Arginine methyl ester . dihydrochloride, which contains the ester group and side chain of trypsin and plasmin substrates, proved somewhat inhibitory. However, all other compounds tested except benzoyl-L-arginine appeared to have no significant effect on the hydrolysis of TAME. The lack of action observed for *p*-chloromercuribenzoic acid indicates that SH group(s) are not essential for enzyme activity. The inhibition by benzoyl-L-arginine exhibited toward plasmin 1E and trypsin under these conditions, wherein TAME is the substrate, takes on an interesting significance in light of the lack of inhibition of trypsin by

TABLE III

STUDY OF THE INHIBITION OF PLASMIN 1E BY VARIOUS ORGANIC AGENTS: SOME COMPARISONS WITH TRYPSIN AT EQUAL ACTIVITY CONCENTRATION

Common conditions.—$S_0$: 0.01 *M* TAME; temp.: 38° C.; pH: 8.05 ± 0.02; buffer: 0.015 *M* veronal.
Plasmin 1E, $E_0$: 0.097 mgm. N/ml.; trypsin, $E_0$: 0.00018 mgm. N/ml.

| Organic agent (0.01 *M*) | Relative activity[a] | |
|---|---|---|
| | Plasmin 1E | Trypsin |
| None | 1.00 | 1.00 |
| Taurine | 1.23 | 1.00 |
| L-Arginine methyl ester$(HCl)_2$ | 0.88 | |
| L-Arginine hydrochloride | 1.06 | |
| $N^{\alpha}$-*p*-Toluenesulphonyl-L-arginine | 0.99 | |
| N-*p*-Toluenesulphonylglycine | 1.00 | |
| *p*-Chloromercuribenzoic acid | 0.98 | |
| $N^{\alpha}$-*m*-Nitrobenzoyl-L-arginine | 0.97 | |
| $N^{\alpha}$-Benzoyl-L-arginine | 0.82 | 0.70 |

[a] *At 15 min. of reaction.*

benzoyl-L-arginine observed here and by Schwert and Eisenberg (9) when BAME is the substrate (under identical conditions). This appears to indicate that an amide grouping at the secondary peptide position of a typical trypsin substrate tends to increase the binding capacity of the compound toward the enzyme as compared to a sulphonyl amide grouping at this position. This takes on further interest when we consider that TAME, having, on the other hand, the sulphonyl amide bond at the secondary peptide position, is hydrolyzed considerably more rapidly than BAME, which has an amide bond at this same position.

*Activation of Plasmin 1E by Inorganic Ions*

Three ions, represented by their chlorides, which appear to have an activating influence on the behavior of plasmin 1E are listed in Table IV. The extent of activation of plasmin 1E by calcium ions was of similar magnitude to that observed toward trypsin at an equal activity concentration. The activation by $Mg^{++}$ and $Ba^{++}$ ions is of too small a dimension to be considered of significance. The activation by calcium ion parallels that observed for trypsin by Green and Neurath (1) using benzoylarginine ethyl ester as substrate.

*Inhibition Studies on Plasmin 1E with Inorganic Compounds Showing Little or no Effect*

The data are presented in Table V from which it will be seen that the activity of plasmin 1E is practically unaffected by the presence of any of the compounds listed. The near doubling of the slight inhibitory effect of KCl by $K_2SO_4$ is probably due to the doubling of the potassium ion concentration.

*Inhibition Studies on Plasmin 1E and Trypsin with Inorganic Compounds Showing Significant Effects on Either or Both Enzymes*

Several cations, represented as their chlorides, which showed significant inhibitory qualities towards plasmin 1E are listed in Table VI wherein their

TABLE IV

ACTIVATION OF PLASMIN 1E BY INORGANIC IONS

Common conditions.—$S_0$: 0.01 *M* TAME; temp.: 38° C.; pH: 8.05 ± 0.02; buffer 0.015 *M* veronal.

Plasmin 1E, $E_0$: 0.097 mgm. N/ml.; trypsin (conc. A): 0.00018 mgm. N/ml.; trypsin (conc. B): 0.00035 mgm. N/ml.

| Salt (0.01 *M*) | Relative activity | | |
|---|---|---|---|
| | Plasmin 1E[a] | Trypsin[a] (conc. A) | Trypsin[b] (conc. B) |
| None | 1.00 | 1.00 | 1.00 |
| $CaCl_2$ | 1.23 | 1.19 | 1.11 |
| $MgCl_2$ | 1.09 | — | — |
| $BaCl_2$ | 1.06 | — | — |

[a] *At 15 min. of reaction.*
[b] *At 8 min. of reaction.*

inhibitory qualities toward trypsin are also recorded. It is significant that in all cases, except that of $NiCl_2$, the ions are more potent in their inhibition toward trypsin (at concentration A) than toward the equivalent activity concentration of plasmin 1E. That the reversal of this trend takes place with $NiCl_2$ is a strong argument in favor of the thesis that plasmin 1E, though activated by trypsin from its proenzyme form, is an individual and distinct enzyme but possesses properties remarkably similar to trypsin. If one

TABLE V

INHIBITION OF PLASMIN 1E—INORGANIC COMPOUNDS WITH LITTLE OR NO EFFECT

Common conditions.—$S_0$: 0.01 *M* TAME; temp.: 38° C.; pH: 8.05 ± 0.02; buffer: 0.015 *M* veronal; $E_0$: 0.097 mgm. N/ml.

| Salt | Salt conc., *M* | Relative activity[a] |
|---|---|---|
| None | | 1.00 |
| KCl | 0.01 | 0.96 |
| $K_2SO_4$ | 0.01 | 0.88 |
| $FeCl_2$[b,c] | 0.001[d] | 0.96 |
| $FeCl_3$[c] | 0.001[d] | 0.94 |
| $PbCl_2$ | 0.0005[d] | 0.86 |

[a] *At 15 min. of reaction.*
[b] *The ferrous ion is rapidly converted to ferric at the reaction pH.*
[c] *Slight $Fe(OH)_3$ precipitation noted at the end.*
[d] *This concentration is about the maximum that will yield a clear starting solution.*

TABLE VI

COMPARATIVE STUDY OF THE INHIBITION OF PLASMIN 1E AND TRYPSIN: INORGANIC COMPOUNDS WITH SIGNIFICANT POTENCE ON EITHER OR BOTH ENZYMES

Common conditions.—$S_0$: 0.01 *M* TAME; temp.: 38° C.; pH: 8.05 ± 0.02; buffer: 0.015 *M* veronal.

Plasmin 1E, $E_0$: 0.097 mgm. N/ml.; trypsin (conc. A), $E_0$: 0.00018 mgm. N/ml.; trypsin (conc. B), $E_0$: 0.00035 mgm. N/ml.

| | | Relative activity | | |
|---|---|---|---|---|
| Salt | Salt conc.,[a] *M* | Plasmin 1E[b] | Trypsin[b] (conc. A) | Trypsin[c] (conc. B) |
| None | | 1.00 | 1.00 | 1.00 |
| NaCN | 0.01 | 1.06 | 0.77 | |
| $AgNO_3$ | 0.0005 | 1.00 | 0.64 | |
| $HgCl_2$ | 0.00075 | 0.82 | 0.52 | |
| $HgCl_2$ | 0.0001 | 0.97 | 0.88 | |
| $NiCl_2$ | 0.001 | 0.42 | 0.58 | 0.58 |
| $ZnCl_2$ | 0.001 | 0.41 | 0.12 | 0.10 |
| $CoCl_2$ | 0.0005 | 0.85 | 0.64 | 0.87 |
| $CdCl_2$ | 0.001 | 0.64 | 0.53 | 0.64 |

[a] *These concentrations were compatible with clear ES mixtures at the end of the reactions. In most cases they represent the maximum concentrations that will yield clear solutions.*
[b] *At 15 min. of reaction.*
[c] *At 8 min. of reaction.*

assumed that the activity of the plasmin 1E is due to trypsin, and that the lesser inhibition effects of these ions toward plasmin 1E as compared to trypsin alone is due to protection of the trypsin in the plasmin 1E preparation by what would therefore be a considerably large contamination with non-enzymatic protein of the plasmin 1E preparation, then one would be at a total loss to explain why in the presence of this "protecting protein" the trypsin of plasmin 1E should be more susceptible to $NiCl_2$ than when the trypsin is alone. It is noteworthy that trypsin at concentration B (twice concentration A) is inhibited in a relatively equal manner by both $NiCl_2$ and $ZnCl_2$, whereas the inhibition by $CoCl_2$ and $CdCl_2$ appears to be related to concentration. This might indicate a difference in the nature of the inhibition by these cations. Also, mercuric ion in its inhibition toward both plasmin 1E and trypsin appears to be dependent upon concentration. These data are in part confirmatory and in part conflicting with those of Green and Neurath (1) and also with those of Grob (2). Whereas Green and Neurath report $Cd^{++}$ and $Co^{++}$ ions to be activating toward trypsin, the data of Table VI show them to be inhibitory toward trypsin and plasmin 1E at the same concentrations as used by these authors. The inhibition of $Zn^{++}$ ions is greater in these studies than that observed by the authors mentioned above. On the other hand, mercuric ion and argentous ion are less inhibitory in this work than in that of the above authors. However, concentrations of these ions are somewhat lower here. It was observed in this work that higher concentrations than those listed in Table VI usually gave rise to precipitates of the corresponding metal oxides and hydroxides and that mercuric and argentous "ions" when used at higher concentrations become extremely potent inhibitors. There is a very strong possibility that this inhibition potency is in great measure due to the removal of the enzyme by adsorption on the precipitating hydroxides. Another reason for the difference observed here as compared to the work of the above authors may be found in the fact that their substrate was benzoylarginine ethyl ester, while that in this work was TAME. Also, buffer and other conditions differ somewhat.

*Inhibition of Plasmin 1E and Trypsin in the Presence of 0.001 M $CuCl_2$*

As can be seen from Table VII, cupric ion is a potent inhibitor of plasmin 1E (Case 3) and virtually eliminates the activity of trypsin (Case 4) at an equal activity enzyme concentration. This cupric ion activity toward trypsin is in agreement with that reported by Green and Neurath (1). It will be observed from Table VII that trypsin incubated for five minutes in the substrate–inhibitor solution prior to the addition of the plasminogen is wholly inhibited (Case 12) thereby losing its ability to activate plasminogen. Similarly, the reaction (Case 10) in which trypsin is so treated and followed by addition of plasmin 1E to the solution yields a relative activity value practically equal to that of plasmin 1E acting alone (Case 3). Since the nitrogen contents of the plasmin 1E and plasminogen preparations are for all practical purposes equal, it is reasonable to conclude that plasmin 1E operates

in the presence of cupric ion and trypsin (Case 10) as a distinct enzyme different but similar to trypsin. Further, it will be noted that, when plasmin 1E and trypsin (concentration A) are added immediately following each other (Case 6) or previously mixed and added together to the substrate–inhibitor solution (Case 7), there is obtained in both cases a value of additional relative activity which is equivalent to *one-third* of the activity of plasmin 1E acting alone (Case 3). Since this extra activity can only be ascribed to trypsin (which has an equal activity concentration with plasmin 1E in the absence of inhibitor (Case 1) ), it would be difficult to explain *two-thirds* of the relative rate observed when plasmin 1E acts alone in the presence of the inhibitor (Case 3) if one makes the assumption that the activity of plasmin 1E is due to the original trypsin which was used to activate it. Thus, at least two-thirds of the activity of plasmin 1E must be due to a distinct enzyme, different but similar to trypsin. The remaining one-third activity of the plasmin 1E preparation in the case of plasmin 1E and trypsin acting in unison (Cases 6 and 7) cannot possibly be ascribed to trypsin since this would represent a trypsin activity concentration equal to the entire activity of the plasmin 1E preparation which is, as shown above, not possible. Consequently, the portion of the remaining one-third activity of the plasmin 1E preparation that may

TABLE VII

COMPARATIVE INHIBITION STUDIES OF PLASMIN 1E AND TRYPSIN, SINGLY OR IN COMBINATION WITH EACH OTHER OR WITH PLASMINOGEN, *in the presence* OF 0.001 *M* $CuCl_2$

Common conditions.—$S_0$: 0.01 *M* TAME; temp.: 38° C.; pH: 8.05 ± 0.02; buffer: 0.015 *M* veronal; plasmin 1E, $E_0$: 0.097 mgm. N/ml.; trypsin (conc. A), $E_0$: 0.00018 mgm. N/ml.; trypsin (conc. B), $E_0$: 0.00035 mgm. N/ml.; plasminogen: 0.104 mgm. N/ml.

| Case No. | Reactants | Experimental procedure | Relative activity[a] |
|---|---|---|---|
| 1 | Plasmin 1E or trypsin (conc. A) only | | 1.00 |
| 2 | Trypsin (conc. B) | | 1.00[b] |
| 3 | Plasmin 1E plus $CuCl_2$ | Enzyme added to substrate–inhibitor solution | 0.31 |
| 4 | Trypsin (conc. A) plus $CuCl_2$ | Enzyme added to substrate–inhibitor solution | 0.03 |
| 5 | Trypsin (conc. B) plus $CuCl_2$ | Enzyme added to substrate–inhibitor solution | 0.05[b] |
| 6 | Plasmin 1E, trypsin (conc. A) plus $CuCl_2$ | Plasmin 1E added to substrate–inhibitor solution followed immediately by trypsin | 0.42 |
| 7 | Plasmin 1E, trypsin (conc. A) plus $CuCl_2$ | Plasmin 1E and trypsin mixed prior to addition to substrate–inhibitor solution | 0.39 |
| 8 | Plasmin 1E, trypsin (conc. B) plus $CuCl_2$ | Plasmin 1E added to substrate–inhibitor solution followed immediately by trypsin | 0.35[b] |
| 9 | Plasmin 1E plus $CuCl_2$ | Enzyme added to substrate–inhibitor solution | 0.18[b] |
| 10 | Plasmin 1E, trypsin (conc. A) plus $CuCl_2$ | Trypsin incubated in substrate–inhibitor solution for 5 min. prior to the addition of plasmin 1E | 0.32 |
| 11 | Trypsin (conc. A), plasminogen 11M54[c] and $CuCl_2$ | Plasminogen added to substrate–inhibitor solution followed immediately by trypsin | 0.36 |
| 12 | Trypsin (conc. A), plasminogen 11M54[c] and $CuCl_2$ | Trypsin incubated in substrate–inhibitor solution for 5 min. prior to the addition of plasminogen | 0.04 |

[a] *Unless otherwise noted, this value taken at 15 min. of reaction.*
[b] *At 8 min. of reaction.*
[c] *Shown to completely lack proteolytic activity.*

be due to trypsin contamination must be rather small. If we assume that two-thirds of the remaining one-third activity of the plasmin 1E is due to the same distinct enzyme which is certainly responsible for two-thirds of the total activity of plasmin 1E, then the trypsin contamination can only be about 10% of the total, *if it exists at all!*

We are also provided in Table VII with a measure of the magnitude of plasminogen activation by trypsin in the presence of cupric ion (Case 11). To be noted, too, is that plasmin 1E, acting alone, yields a value after eight minutes of reaction (Case 9) which, when subtracted from the value observed for the action of plasmin 1E and trypsin (concentration B) (Case 8), yields a value of trypsin action which is approximately twice the value of trypsin action found for plasmin 1E and trypsin (concentration A) (Cases 6 or 7). This indicates that cupric inhibition of trypsin is proportional to concentration.

*Summary of Arguments to Support the Thesis that Plasmin 1E and Trypsin are Distinct Enzymes with Similar Hydrolytic Properties*

(1) The ratio of activities on various substrates is different.

(2) The individual stability curves at pH 8 are different.

(3) The increase in activity observed when trypsin and plasmin 1E or when trypsin and plasminogen are acting in unison (as compared to the sums of the activity of the enzymes acting alone) can lead to no other conclusion but that another similar, though not identical, enzyme is being produced.

(4) Trypsin is soluble at the isoelectric precipitation point of plasmin and plasminogen, though admittedly this does not exclude coprecipitation or trypsin adsorption on the precipitating plasmin preparation proteins.

(5) The reversal of inhibitory potency toward these two enzymes observed in the case of $NiCl_2$ as compared to the other inorganic ions tested points to their non-identity.

(6) Studies on the activity of plasmin 1E and trypsin in the presence of cupric ion stress the individuality of these two enzymes.

*The Plasmin ES Complex*

The behavior of plasmin 1E as described herein in no way conflicts with the previously proposed dipositive-bond theory of hydrolytic enzyme action (6–8). Therefore, it is suggested, in view of the marked similarity between plasmin 1E and trypsin, that plasmin 1E forms the very same type of ES complex as previously assigned to trypsin (7). The difference between the two enzymes is probably a result of a difference in the nature of one or all of the enzyme groups involved in the ES complex; or a difference in the geometry of the active center and its environs; or some combination of these factors.

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# INHIBITION OF AMINE OXIDASE BY METAL IONS AND BY SULPHYDRYL COMPOUNDS[1]

By J. R. Lagnado and T. L. Sourkes[2]

## Abstract

Metal compounds were studied for possible effects on the amine oxidase system of rat liver. Some activated, others inhibited this system. Certain compounds exhibited both actions, the former being detectable at the lower concentrations. The data obtained extend the evidence of others for amine oxidase as a sulphydryl enzyme, but other mechanisms for metal inhibition of amine oxidase must also be considered. The actions of sulphydryl compounds on amine oxidase activity were investigated. BAL, thioglycollic acid, cysteine, and cystine were inhibitors, glutathione activated the enzyme system under certain conditions. Intravenous administration to rats of mercuric acetate, cadmium chloride, lead acetate, and manganese sulphate, all being *in vitro* inhibitors of amine oxidase, did not affect the enzyme activity of brain and liver in acute experiments.

Knowledge of the properties of amine oxidase has been growing slowly since the discovery of the enzyme almost thirty years ago. Although it has not been purified to any great extent its inhibition by some mercury (8, 18) and arsenic (18) compounds, and the reversal of inhibition by glutathione and cysteine in the presence of cyanide (8), have led to its classification with the sulphydryl enzymes. However, inhibition of the enzyme by metals which can react with sulphydryl groups is not sufficient evidence that they do in fact inhibit the enzyme by this mechanism (cf. 3). It was thought important, therefore, to obtain further data on heavy metal inhibition of amine oxidase. Accordingly, an extended list of metal and metal-containing ions was surveyed for effects on this enzyme. Some of the salts which inhibited strongly *in vitro* were tested for possible inhibitory action on rat liver and brain amine oxidase following intravenous injection.

Because of the presumed sulphydryl "active group" in the enzyme it was considered worthwhile to investigate the actions of some —SH compounds on amine oxidase preparations.

## Methods

### *Treatment of Tissues*

Adult male rats, weighing between 100 and 200 gm., were used in this work. The rats were killed by decapitation. The liver was excised, rinsed in cold tap water, weighed, and ground with a glass or Teflon homogenizing pestle to give a 20% tissue suspension in distilled water (20 gm. tissue plus 80 ml. water).

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*Contribution from the Allan Memorial Institute of Psychiatry, McGill University, Montreal, Quebec. This work was aided by a Federal–Provincial Mental Health Grant and by a grant from the Foundation's Fund for Psychiatric Research, to Dr. R. A. Cleghorn. The data are taken from a thesis presented by J. R. Lagnado to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements for the M.Sc. degree. A preliminary account was presented at the 19th Annual Meeting of the Canadian Physiological Society, October 13–15, 1955, at London, Ontario (14).*

[2]*Mimeographed copies of these data will be made available on request.*

This suspension was dialyzed in a cellophane bag against distilled water at 3° for about 20 hr. The dialyzing medium was changed each hour for the first three hours and once more about three hours before use of the suspension. The purpose of this procedure was to reduce the oxygen uptake in the enzyme blanks by removal of endogenous substrate. Dialysis did not decrease the effect of an added amine on oxygen consumption by the tissue extracts (cf. 13). In some cases, dialysis was carried out against cold, running tap water and here, too, no decrease in the rate of oxygen consumption was observed compared with non-dialyzed portions of the same liver suspension. Because some of the compounds to be tested might have effects not only on amine oxidase but also on the complex of enzymes responsible for the endogenous oxygen uptake, it was considered important to use preparations having as low an endogenous rate as possible.

For the *in vivo* experiments, rats weighing 145–160 gm. were selected for a given assay. They were injected intravenously (tail vein) with saline, 0.4 ml./kgm. body weight (controls), or with the same volume of an aqueous solution of the salt under test. Doses of the salts are listed in the text. The animals were sacrificed 0.5–2.0 hr., in various experiments, after the injection. Brain and liver were removed at once and ground in water. If space on the Warburg apparatus was limited the brains were examined first and the livers (stored at 4°) within two to three hours.

*Reaction Mixtures*

*Iso*amylamine, tyramine hydrochloride, and tryptamine hydrochloride were used as substrates. They were made up in aqueous solution, adjusted to pH 7.4 (bromthymol blue) before addition to the Warburg vessels in amounts sufficient to yield a final concentration of 0.02 molar (*M*) in the reaction mixture. At this concentration the enzyme is saturated with substrate (6, 13) and further increments in the substrate concentration do not increase amine oxidase activity. The salts under test were added to the main well, and were thus incubated with the enzyme preparation about 15 min. before addition of substrate.

In most of the experiments tris(hydroxymethyl)aminomethane buffer (Tris), pH 7.4 (0.037 *M* final concentration), was used. The use of phosphate buffer (0.02 *M* final concentration) was limited to only a few experiments, as indicated in the text, because phosphate forms complexes with a number of the metal compounds studied and has even been suggested to have a slight stimulating effect on amine oxidase (12).

Oxygen uptake was measured by Warburg's direct method (21). Thirty per cent KOH solution was placed in the center well. Substrate was pipetted into the side arm and was poured into the main compartment containing the other ingredients after it was gassed with oxygen and the flasks were equilibrated to 38°. In reaction mixtures like this, which include crude liver preparations, there may occur other reactions which affect the net oxygen uptake. In many of the experiments *in vitro* no precautions were taken to

exclude reactions other than the oxidation of the amine to the corresponding aldehyde because the matter of first interest was the determination of the effects of the metals on the system of enzymes involved in the intermediary metabolism of amines. In the case of amine oxidase this procedure was experimentally justified by the fact that most of the oxygen consumed in the oxidation of tryptamine, for example, under the above conditions, is taken up in the first reaction, i.e., aldehyde formation (20). All salts which inhibited oxidation in such reaction mixtures were later retested in the presence of $2 \times 10^{-2}$ *M* semicarbazide. In the reaction mixtures prepared for suspensions of tissues from the intravenously injected animals, semicarbazide ($10^{-2}$ *M*) was also employed as an aldehyde trapping agent. In these experiments the rate of oxygen uptake was constant for at least 30 min.

In some experiments, the determination of amine oxidase activity was performed by the method of substrate disappearance (20).

## Results

*Effects of Metal Salts in vitro*

At the start of this work, it was found that the following compounds in 0.01 *M* concentration have no effect on the oxidation of tyramine, a typical substrate for amine oxidase: KCl, $K_2SO_4$, NaCl, $Na_2SO_4$, $NH_4Cl$. In addition to this group, the following compounds tested in concentrations as high as those indicated in parenthesis were without effect on the amine oxidase system of rat liver: $TiOSO_4$ ($4 \times 10^{-3}$ *M*), $Ce(SO_4)_2$ ($5 \times 10^{-3}$ *M*), $Na_2SeO_3$ ($10^{-2}$ *M*), and $BaCl_2$ ($10^{-3}$ *M*).

Table I summarizes some of the data obtained. Several concentrations of each salt were tested and from the results obtained the concentrations corresponding to rates of oxidation (extra $\mu$l. $O_2$/30 min. due to the addition of substrate) equal to 20, 50, and 80% respectively, of the control mixtures, were estimated graphically. Many additional experiments not listed here were performed in which only one or two concentrations of a particular salt were tested and the results were used to guide the choice of concentrations in the subsequent experiments. The results with reaction mixtures which contained semicarbazide are given in the right-hand column of Table I. In the presence of semicarbazide endogenous respiration frequently rose above blank values. This was noted with the addition of aluminum, cadmium, calcium, cerium, copper, iron, selenium, silver, and zinc salts.

In the absence of added substrate and semicarbazide, oxygen uptake was markedly activated by ferric salts in concentrations of the order of $10^{-4}$ *M* or higher. With the addition of both substrate and ferric ions the oxygen uptake never exceeded that of the control mixtures (Table II). Pigment formation nevertheless occurred when tyramine or tryptamine was used, and this may be taken as an indication that the amines are oxidized to some extent (17). The activating effect of ferric salts on the endogenous respiration was observed when incubations were carried out under both air and oxygen. It is

TABLE I

INHIBITION OF AMINE OXIDASE BY SALTS OF METALS

| Compound | Expt. No. | −Log (concentration) to inhibit 20% | 50% | 80% | Effect of $10^{-3}$ *M* salt* % of control |
|---|---|---|---|---|---|
| Mercuric acetate | 1 | 3.77 | 3.44 | 3.35 | |
| | 2 | | | 3.40 | |
| Silver nitrate | 1 | 3.62 | 3.37 | 3.12 | 80 |
| | 2 | 3.40 | 3.00 | 2.72 | 77 |
| | 3 | 3.96 | 3.60 | 3.22 | 18 |
| Ammonium molybdate | 1 | 3.80 | 3.56 | 3.31 | 36 |
| | 2 | 3.72 | 3.35 | 2.95 | |
| | 3 | 3.65 | 3.26 | | |
| | 4 | 3.26 | 3.13 | 2.86 | |
| Cadmium chloride | 1 | 3.00 | | | 61 |
| | 2 | 3.70 | 3.40 | 3.08 | |
| Aluminum sulphate | | | 3.15 | | 3 |
| | | | | | 38 |
| Lead acetate | | 3.50 | | | 100 |
| Copper sulphate | | 3.20 | 2.72 | 2.25 | 7 |
| | | | | | 32 |
| Potassium permanganate | 1 | 2.55† | | | |
| | 2 | | 2.88 | 2.30 | |
| Sodium dichromate | 1 | 2.60 | 1.94 | | |
| | 2 | 3.04 | 1.94 | | |
| Potassium chromate | | 2.30§ | | | |
| Uranyl acetate | 1 | 2.00 | 1.77 | 1.55 | 76 |
| | 2 | | | 1.70 | |
| Cuprous chloride | 1 | | 1.30 | | 0 |
| | 2 | 2.90 | 2.22 | 1.92 | |
| Manganese sulphate | 1 | 3.00 | | | 75 |
| | 2 | 2.00† | | | |
| | 3 | 2.50 | | | |
| Magnesium sulphate | | 1.75‡ | | | 113 |
| Calcium chloride | 1 | 2.70§ | | | |
| | 2 | 3.00§ | | | |
| Selenium oxide | | 2.30§ | | | |
| Zinc sulphate | | | | 3.00‖ | 86 |
| Barium chloride | | 2.00‡ | | | |
| Nickelous chloride | | 3.00¶ | | | |

*In presence of $2 \times 10^{-3}$ *M* semicarbazide.
†30% inhibition.
‡25% inhibition.
§10% inhibition.
‖Complete inhibition.
¶Result observed in three experiments.

TABLE II

EFFECTS OF FERRIC SALTS ON AMINE OXIDASE ACTIVITY OF RAT LIVER SUSPENSIONS

Reaction mixtures as described under "Methods". Ferric chloride used in Expts. 1, 3, and 4; ferric sulphate in Expt. 2. Amounts of dialyzed liver suspension used corresponded to 100 mgm. wet wt. of tissue. Gas phase, $O_2$ in Expts. 1–3, air in Expts. 4 and 5

| Expt. No. | Final conc. of $Fe^{3+}$, M./l. | Oxygen consumption, $\mu$l./30 min. | | |
|---|---|---|---|---|
| | | Endogenous | Tyramine | Tryptamine |
| 1 | | 33 | 114 | |
| | $2.5 \times 10^{-2}$ | 105 | 105 | |
| 2* | | 33 | 114 | 100 |
| | $5.0 \times 10^{-5}$ | 42 | 114 | |
| | $2.5 \times 10^{-2}$ | 178 | 61 | 112 |
| 3 | | 0 | 198 | |
| | $10^{-4}$ | 184 | 170 | |
| 4 | | 0 | 30 | 39 |
| | $10^{-4}$ | 158 | 123 | 123 |
| 5 | | 0 | 35 | |
| | $10^{-4}$ | 180 | 178 | |

**The liver suspension was not dialyzed for this experiment.*

possible that the increased oxygen uptakes are due to a peroxidatic reaction for which the ferric salts are known to act as catalysts (15). Ferrous sulphate in concentrations up to $10^{-3}$ *M* was without effect on amine oxidase.

Several compounds enhanced oxygen uptake of the liver preparations in the presence of an amine. Examples of this activation are given in Table III. In higher concentrations some of these compounds proved to be inhibitory.

*Effects of Intravenously Administered Salts*

Four of the salts which inhibited amine oxidation *in vitro* were selected for tests *in vivo*. The compounds and dosages used in the several tests were as follows: mercuric acetate (40 and 200 mgm./kgm. body weight, 8 animals); cadmium chloride (200 mgm./kgm., 10 animals); manganese sulphate (200, 400, 500, and 1000 mgm./kgm., 40 animals); lead acetate (500 and 1000 mgm./kgm., 8 animals). Because these metals cause neuropsychiatric symptoms, at least on chronic intake regimes (19), it was of interest to determine whether they affected amine oxidase activity of brain as well as of liver when administered in acute experiments. Rats were injected with various salts in the dosages listed above. Control animals were similarly injected with saline. The animals were killed 0.5–2.0 hr. after the injections were performed. Brain and liver were excised and prepared in the usual way as suspensions in water for the determination of amine oxidase. It was then found that none of the salts tested affect significantly the amine oxidase activity of the tissues. That the doses were sufficiently high to cause toxic

TABLE III

ACTIVATION OF AMINE OXIDASE BY METAL IONS*

| Compound | Final conc., M./l. | % of control value |
|---|---|---|
| Cuprous chloride | $10^{-6}$ | 120 |
| Cupric sulphate | $2.5 \times 10^{-5}$ | 133 |
| Cupric sulphate | $10^{-4}$ | 108 |
| Silver nitrate | $10^{-4}$ | 112 |
| Cadmium chloride | $10^{-4}$ | 138 |
| Mercuric acetate | $5.0 \times 10^{-7}$ | 115 |
| Mercuric acetate | $5.0 \times 10^{-6}$ | 142 |
| Stannous chloride | $5.0 \times 10^{-3}$ | 112 |
| Sodium selenite | $5.0 \times 10^{-6}$ | 108 |
| Uranyl acetate | $5.0 \times 10^{-6}$ | 149 |
| Cobaltous nitrate | $5.0 \times 10^{-3}$ | 141 |
| Ferrous sulphate | $10^{-6}$ | 116 |
| Ferrous sulphate | $10^{-5}$ | 123 |

**In the experiment with cadmium chloride the substrate was tryptamine. In the others it was tyramine.*

symptoms was demonstrated by the decreased righting reflex and the somnolence exhibited by the rats injected with cadmium chloride; by the decreased tonus of the muscles of the hind limbs following treatment with manganese sulphate, as well as by the marked ataxia and reduced respiration rate on the highest dose; and by intestinal hemorrhages following administration of the mercury salt. The doses of lead acetate used resulted in no obvious symptoms.

*Effects of Sulphydryl Compounds*

*BAL (2,3-dimercaptopropanol).*—In the usual reaction mixtures BAL underwent autoxidation. This reaction is known to be catalyzed by traces of copper, iron, or manganese, metals which form unstable complexes with thiol groups, in contrast to cadmium, mercury, and silver, which form mercaptides with small dissociation constants (1). The oxidation proceeded more rapidly in the presence of liver suspensions so that it is clear that the tissue actually contributes to the oxidation of the added BAL (Table IV). In spite of the high oxygen uptake in the blanks occasioned by the use of BAL, inhibition of amine oxidase by this compound could be observed (Table IV).

*Thioglycollic acid (mercaptoacetic acid, TGA).*—TGA inhibited amine oxidase activity as determined both manometrically and spectrophotometrically (Tables IV and V). Like BAL, it also enhanced the respiration of the tissue blanks, at the higher concentrations tested.

*Cysteine and cystine.*—A high concentration of cysteine inhibited the enzyme, but assurance of inhibitory effects by lower concentrations was vitiated by the acceleration of endogenous respiration due to the cysteine added. For this reason it was thought useful to find what effects dialysis of the tissue against cysteine solution would have on amine oxidase activity. Tissue suspensions were dialyzed six hours against $M/50$ cysteine, pH 8.1,

TABLE IV

EFFECTS OF THIOL COMPOUNDS ON LIVER AMINE OXIDASE

| Expt. No. | Addition | Final conc., M./l. | $\mu$l. $O_2$/30 min. | | |
|---|---|---|---|---|---|
| | | | Endogenous | +Tyramine | Difference |
| 1 | — | | 7 | 111 | 104 |
| | BAL | $4.0 \times 10^{-4}$ | 15 | 109 | 94 |
| | BAL | $8.0 \times 10^{-4}$ | 20 | 76 | 56 |
| 2 | — | | 5 | 100 | 95 |
| | BAL | $4.0 \times 10^{-4}$ | 42 | 114 | 72 |
| | BAL* | $4.0 \times 10^{-4}$ | 21 | 13 | −8 |
| 3 | — | | 0 | 90 | 90 |
| | BAL | $8.0 \times 10^{-3}$ | 152† | 202 | 50 |
| | BAL | $1.6 \times 10^{-2}$ | 232 | 278 | 46 |
| | BAL* | $1.6 \times 10^{-2}$ | 126 | 116 | −10 |
| 4 | — | | 0 | 100 | 100 |
| | TGA | $2.0 \times 10^{-3}$ | 48 | 22 | −26 |
| 5 | — | | 0 | 84 | 84 |
| | TGA | $2.5 \times 10^{-3}$ | 9 | 25 | 16 |
| 6 | — | | 5 | 100 | 95 |
| | TGA | $5.0 \times 10^{-3}$ | 51 | 51 | 0 |
| | TGA* | $5.0 \times 10^{-3}$ | 6 | −3 | −9 |
| 7 | — | | 15 | 99 | 84 |
| | Cysteine | $3.2 \times 10^{-2}$ | 5 | 2 | −3 |
| 8 | — | | 56 | 99 | 43 |
| | Cysteine | $4.0 \times 10^{-3}$ | 85 | 104 | 19 |
| 9 | — | | 20 | 140 | 120 |
| | Cysteine | $2.0 \times 10^{-4}$ | 42 | 152 | 110 |
| | Cysteine | $2.0 \times 10^{-3}$ | 75 | 140 | 65 |
| 10† | — | | 0 | 81 | 81 |
| | Cysteine‡ | $10^{-2}$ | 55 | 87 | 32 |

**Tissue omitted.*
*†Phosphate buffer (pH 7.4 M/50 final conc.) used instead of Tris.*
*‡No inhibition observed in this experiment up to $2 \times 10^{-3}$ M cysteine.*

TABLE V

INHIBITION OF AMINE OXIDASE BY CYSTEINE AND THIOGLYCOLLIC ACID

| Expt. No. | Addition | Final conc., M./l. | Extra $O_2$ uptake, $\mu$M./30 min. | Tryptamine disappearing,* $\mu$M./30 min. |
|---|---|---|---|---|
| 1 | — | | 1.3 | 3.8 |
| | TGA | $4.0 \times 10^{-3}$ | 0.1 | 0.0 |
| 2 | — | | 2.2 | 6.1 |
| | Cysteine | $3.2 \times 10^{-2}$ | 0.0 | 4.3 |

**Method of substrate disappearance (20) used.*

TABLE VI

EFFECTS OF DIALYSIS AGAINST CYSTEINE ON AMINE OXIDASE ACTIVITY OF RAT LIVER SUSPENSIONS

| Expt. No. | Dialysis medium | $\mu$l. $O_2$/30 min. | | |
|---|---|---|---|---|
| | | Endogenous | +Tyramine | Difference |
| 1 | Water | 10 | 125 | 115 |
| | Cysteine | 20 | 85 | 65 |
| 2 | Water | 25 | 165 | 140 |
| | Cysteine | 20 | 120 | 100 |
| 3 | Water | 7 | 122 | 115 |
| | Cysteine | 24 | 107 | 83 |

NOTE: *For procedure, see text. M/50 cysteine solution, pH 8.1, used as dialyzing medium.*

then 12 hr. against distilled water to remove the cysteine. Control samples were dialyzed against water adjusted to pH 8.1 for six hours and then against distilled water for 12 hr. longer. Dialyses were performed at 3°. The data of Table VI indicate that dialysis against cysteine consistently decreased amine oxidase activity, but had little, if any, effect on endogenous respiration. Cystine, on the other hand, had no effect on amine oxidase when added as a suspension. When ground with the tissue in the homogenizing tube, it was able to produce a small inhibitory effect. The low solubility of cystine in water may account for the limited effects seen.

*Glutathione.*—Added GSH (3 mgm.) was found to enhance amine oxidase activity little or not at all when oxygen was used as the gas phase. When the incubations were performed under air, a procedure which reduces amine oxidase activity as measured manometrically, GSH was found to have an activating effect (14, 33, 64%, respectively, in three experiments).

## Discussion

One of the aims of this work was to survey the effects of various metal and metal-containing ions on the enzymatic system of liver which oxidizes monoamines. The results obtained, as summarized in Table I, indicate the possibility of a variety of mechanisms in the inhibition of amine oxidase. For example, if a single mechanism of inhibition were operating, e.g., by mercaptide formation, then all the inhibitory compounds ought to be equally effective, aside from experimental error and a possible correction factor for valence. The latter would probably be small; a divalent cation might be twice as active as a monovalent one, but this would merely reduce the logarithm of the inhibiting concentration by 0.3. In fact, these values (for 50% inhibition of amine oxidase) range from $-3.60$ to $-1.30$, i.e., from below $10^{-3}$ $M$ to about $5 \times 10^{-2}$ $M$ (Table I). Hence, in spite of the evidence for mercaptide formation by compounds of heavy metals in the inhibition of amine oxidase (8, 18), it is not unreasonable to anticipate more than one mechanism.

Inspection of the "50%" Column of Table I shows that the compounds tested appear to fall into three groups, the first of which includes mercury, cadmium, silver, and molybdate. These have values of about −3.40. Aluminum (−3.15) and lead may also be in this group. Mercury, cadmium, and silver are all known to be capable of inhibiting a number of enzymes having functional —SH groups (9). The present results with inorganic mercurial salts confirm the earlier work with organic compounds (8, 18). In this connection, Haarman's findings (11) may be cited as a caution. He found that mercury salts can combine loosely with many amino acids and more firmly with the imidazole ring of histidine, the —SH groups of cysteine, and possibly the peptide bonds in protein. Since added sulphydryl compounds would likely withdraw mercury from such linkages it is evident that "sulphydryl reversal" experiments cannot of themselves prove that the site of action of the mercury was at an —SH group. As with mercury, cadmium inhibition can be reversed by BAL both *in vivo* and *in vitro* (9). The mechanism by which molybdate inhibits is not clear.

The second group of inhibitory salts includes cupric sulphate and potassium permanganate. These are required in greater than $10^{-3}$ *M* concentration to give 50% inhibition of amine oxidase. The results with copper confirm the findings of Bernheim (4, cf. also 16), who suggested that this metal is an —SH inhibitor. In regard to permanganate it is known that free —SH groups are readily oxidized by it at neutral pH (1). It was necessary to employ phosphate buffer with the permanganate, since Tris rapidly decolorizes solutions of the latter compound.

Chromate and dichromate were both tested as possible —SH oxidizing agents, but they were less active than permanganate as inhibitors. They fall into the third category, which also includes uranyl acetate and cuprous chloride, salts which inhibit amine oxidase substantially only above $10^{-2}$ *M*. Uranyl acetate inhibited both tyramine and tryptamine oxidation in the present experiments. Dounce found no effect from it ($5 \times 10^{-4}$ *M*) on the course of adrenaline oxidation (7), a result which is not in disagreement with the present one. Concentrations of uranium acetate above $10^{-3}$ *M* caused a flocculation of proteins in the flasks, so that it is possible that the inhibition is due to enzyme precipitation and not to some specific effect on amine oxidase. Bernheim (5) reported selenite capable of inhibiting by catalyzing the oxidation of —SH groups. In the present work sodium selenite ($10^{-2}$ *M*) was without effect and selenium oxide had only a mildly inhibitory action on the enzyme system.

The compounds which produced an apparent activation did so in concentrations of the order of $10^{-4}$ *M* or lower. Since crude tissue suspensions were used, the "activation" may simply be a differential inhibition of catalase present at concentrations below those required to inhibit the oxidation of the amine. Inhibition of catalase, an enzyme not affected, incidentally, by sulphydryl reagents (2), would result in return of less oxygen from the hydrogen peroxide formed to the gas phase. Whatever the mechanism,

the phenomenon has also been observed by Perry, Teitlebaum, and Schwartz (16) in the case of ferrous, vanadyl, and cobaltous salts, and by Govier *et al.* (10) in the case of aluminum and calcium salts. The possibility of a role for a metal as a cofactor of amine oxidase is, of course, suggested by these results, and cannot be excluded.

The failure to detect any action *in vivo* of salts of mercury, manganese, cadmium, and lead on amine oxidase may well signify that this enzyme is not affected during the development of the acute symptoms. The case may be different in chronic toxicity, where long-continued ingestion or inhalation of the toxic compound, as in occupational poisoning (19), can seriously affect the neuropsychiatric status of the subject.

The inhibitory action of the —SH compounds BAL, TGA, and cysteine, and of the disulphides tested on amine oxidase is consonant with the concept of this enzyme as possessing a sulphydryl group functioning reversibly in an oxidation–reduction process. Such an enzyme should be capable of being inhibited either in the reduced or in the oxidized phase of its functional cycle by an excess of sulphydryl or disulphide compound, respectively. Glutathione, however, activated the enzyme system when the oxidation was performed under air. How this fact fits into the above concept remains to be determined by more detailed experiments with this series of compound.

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# EXTRACELLULAR ORGANIC NITROGEN IN USTILAGO MAYDIS FERMENTATION BROTHS[1]

By Eugene L. Dulaney,[2] E. Bilinski,[2] and W. B. McConnell

## Abstract

Free amino acids and small peptides make up most of the extracellular organic nitrogen in media from shaken and aerated *Ustilago maydis* fermentations. Of the 3.5 mgm./ml. ammonia nitrogen added, 2.9 mgm./ml. remained in the extracellular broth. This extracellular nitrogen contained 1.17 mgm./ml. of organic nitrogen and 1.74 mgm./ml. of residual ammonia nitrogen. At least 53% of extracellular organic nitrogen is in the form of free amino acids. Fifteen amino acids were estimated quantitatively in acid-hydrolyzed broth and a particularly high level of arginine (1.14 mgm./ml.) was found. The amounts of methionine and tryptophan in the broth were quite low but the lysine concentration 0.400 mgm./ml. was relatively high.

## Introduction

The production of feed supplements rich in certain essential amino acids would be of great value to the livestock industry. While chemical synthesis of some acids may be approaching economic levels, microbial synthesis would have the advantage of yielding the naturally-occurring isomers. Two alternatives are possible, namely the build-up of cellular proteins of unusual composition or the synthesis and secretion of amino acids or simple peptides into the fermentation medium by the organism. From previous experience in this laboratory on the production of extracellular proteases (1) the latter possibility appeared to offer the greatest potentialities. Accordingly Richards and Haskins (7) assayed and selected several strains of fungi which produced unusually high levels of lysine in the medium. Dulaney (2) using the most promising cultures then studied their nutrition and tried to arrive at optimal conditions for the production of lysine. It seemed advisable before conducting feeding trials on these broths to obtain more detailed information on the over-all nitrogen picture and, in so far as possible, a comparison of the levels of all the essential amino acids present.

In this communication the results of a study of the extracellular nitrogen compounds in *Ustilago maydis* fermentation broths are reported.

## Materials and Methods

The culture used in the present work is a strain of *Ustilago maydis* (DC.) Cda. It was one of a group of cultures selected by Richards of this laboratory in a search for fungi capable of producing large amounts of extracellular free lysine (7) and has been used in experimental studies on the formation of extracellular lysine (2).

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[2]*National Research Council of Canada Postdoctorate Fellow 1955–1956.*

The culture was grown in 250 ml. Erlenmeyer flasks containing 50 ml. of the following medium: *d*(+)dextrose 100 gm., $(NH_4)_2HPO_4$ 16.52 gm. (3.5 mgm. N/ml.), $K_2HPO_4$ 0.3 gm., $KH_2PO_4$ 0.2 gm., $MgSO_4 \cdot 7H_2O$ 0.2 gm., NaCl 0.2 gm., $CaCl \cdot 2H_2O$ 0.2 gm., $FeSO_4 \cdot 7H_2O$ 0.015 gm., $ZnSO_4 \cdot 7H_2O$ 0.01 gm., and distilled $H_2O$ to 1 liter. The $(NH_4)_2HPO_4$ was sterilized separately. The initial pH was 7.0. After inoculation, the flasks were incubated at 27°–28° C. on a rotary shaker moving at 225 r.p.m. and describing a circle 2 in. in diameter. After 100 hr. growth, when the extracellular free lysine was near the highest level, the cells were centrifuged from the broth.

The total nitrogen in the unhydrolyzed broth was measured by micro-Kjeldahl determination and a value termed organic nitrogen was obtained by repeating the Kjeldahl determination after removal of ammonia. Ammonia nitrogen was estimated by difference. The amino nitrogen of free amino acids in the unhydrolyzed broth was determined with good agreement by each of the two ninhydrin decarboxylation procedures (3, 9). A portion of the unhydrolyzed broth was assayed for lysine, aspartic acid, serine, proline, glutamic acid, isoleucine, leucine, tryosine, phenylalanine, glycine, and methionine, using *Leuconostoc mesenteroides*, P-60, ATCC No. 8042. *Lactobacillus arabinosus* No. 17-5 was used to assay for valine and tryptophan.

The free lysine, histidine, and arginine in the broth were isolated by chromatography on 15 cm. columns of Dowex-50, according to the method of Moore and Stein (4).

A portion of the broth was hydrolyzed by being refluxed with 6 *N* HCl for 24 hr. and the free amino acids in the resultant hydrolyzate determined by chromatography on a 150 cm. column of Dowex-50. The gradient elution technique was employed (5) and all effluent fractions were analyzed by the modified ninhydrin method of Moore and Stein (6).

The amino acids were identified in the usual manner according to their time and order of elution. Except for some overlapping of the glycine and alanine fractions a complete separation of all the ninhydrin positive compounds in the hydrolyzate was achieved. One unidentified compound corresponding closely to ornithine in elution position was observed but no further identification was attempted. Its color yield was equivalent to an ornithine concentration of 127 μgm./ml. Lysine and arginine after elution were further identified. The eluants were desalted (8) and rechromatographed on paper using mixed chromatograms.

## Results and Discussion

Eighty-three per cent of the nitrogen added (3.5 mgm. N/ml.) remained in the broth and the rest apparently was transformed into cellular nitrogenous compounds. A complete nitrogen balance was not obtained but it is doubtful if much ammonia is lost through diffusion as the pH of the broth falls from pH 7.0 to below 3.0.

The extracellular nitrogen contained 60% unconverted ammonia nitrogen (1.74 mgm./ml.) and 40% organic nitrogen (1.17 mgm./ml.).

TABLE I

AMINO ACID COMPOSITION OF *U. maydis* FERMENTATION BROTH

| Amino acid* | Unhydrolyzed broth | | Hydrolyzed broth | |
|---|---|---|---|---|
| | μgm./ml. | μgm. N/ml. | μgm./ml.† | μgm. N/ml.† |
| Lysine | 387 | 64.2 | 413 | 79.2 |
| Arginine | 997 | 320.5 | 1136 | 365.3 |
| Histidine | 155 | 42.0 | 182 | 49.3 |
| Aspartic acid | 200 | 21.0 | 506 | 53.2 |
| Glutamic acid | 894 | 85.1 | 945 | 90.0 |
| Glycine | 200 | 38.1 | 295 | 55.0 |
| Alanine‡ | | | 406 | 63.8 |
| Valine | 290 | 25.7 | 279 | 33.4 |
| Leucine | 387 | 41.3 | 368 | 39.3 |
| Isoleucine | 276 | 29.5 | 212 | 22.7 |
| Serine | 276 | 30.1 | 307 | 40.9 |
| Threonine‡ | | | 237 | 27.9 |
| Proline | 263 | 32.0 | 289 | 35.2 |
| Phenylalanine | 267 | 22.6 | 389 | 33.0 |
| Tyrosine | 139 | 10.7 | 383 | 29.6 |
| Tryptophan | 40 | 5.6 | | |
| Methionine | 65 | 6.0 | | |
| | 4.79 mgm. | 0.774 mgm. | 6.35 mgm. | 1.02 mgm. |

**The amino acids in the hydrolyzed broth and the basic amino acids in the unhydrolyzed broth were separated chromatographically and assayed colorimetrically. The other amino acids in the unhydrolyzed broth were assayed microbially.*

*†Values expressed as μgm./ml. in terms of original broth.*

*‡Valid microbial assays were not obtained.*

The quantities of amino acids in the organic nitrogen portion of hydrolyzed and unhydrolyzed broth are listed in Table I.

Fifteen amino acids were isolated from the hydrolyzed broth by chromatography. Since tryptophan is destroyed by hydrolysis and since cystine, cysteine, and methionine are partially oxidized during chromatography, estimates of these amino acids were not attempted. Both methionine and tryptophan were found to be present at low levels in the unhydrolyzed broth by microbial assay.

A large percentage of the organic nitrogen in the unhydrolyzed broth is represented by free amino acids. The $\alpha$-amino nitrogen of free acids is 0.31 mgm./ml. and by adding to this the additional nitrogen in arginine, lysine, histidine, and tryptophan, a value for nitrogen in free amino acids of 0.62 mgm./ml. is obtained. Thus 53% of the extracellular organic nitrogen is represented by free amino acids. Microbial assays of the unhydrolyzed broth confirm the presence of large amounts of free amino acids. It is appreciated that these assay organisms may respond in part to amino acids in small peptides as well as to free amino acids; however, based on these microbial

assays, 76% of the extracellular organic nitrogen may be in the form of free amino acids or small peptides. Values for alanine and threonine were not obtained microbially and are not included in these calculations. Obviously the bulk of the extracellular organic nitrogen is in the form of free amino acids or small peptides. It is not clear just how these free amino acids and peptides are formed but they are probably not the product of protein hydrolysis as protein hydrolyzates would be expected to contain a distribution of amino acids quite different from that observed.

The low levels of methionine and tryptophan in this broth are disappointing. A culture that makes relatively high concentrations of extracellular tryptophan is needed because acid hydrolysis of cellular proteins cannot be depended upon to yield this amino acid. The results, however, do indicate that microbial fermentations are a promising source of amino acid feed supplements.

## Acknowledgments

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# CONVERSION OF PROTHROMBIN TO AUTOPROTHROMBIN II[1]

By John A. Penner, F. Duckert, Shirley A. Johnson, and Walter H. Seegers

## Abstract

With thromboplastin, Ac-globulin, and calcium ions, purified prothrombin converts to biothrombin. This derivative of prothrombin is also obtained with purified platelet factor 3, Ac-globulin, calcium ions, and autoprothrombin II. Need for the latter substance by platelet factor 3 is one marked distinction that can be made between thromboplastin and platelet factor 3. Thromboplastin does not require a cofactor such as platelet cofactor I or autoprothrombin II. Ether extracts of dry brain or of purified platelet factor 3 or of whole platelets yield lipid material. The lipids obtained in this manner are active in a modified Biggs and Douglas thromboplastin generation test; but in contrast to the original thromboplastin or purified platelet factor 3 from which they are obtained do not activate purified prothrombin to thrombin. The lipid material is thus distinctly different from thromboplastin or from platelet factor 3. The lipid is also active in the conversion of prothrombin to autoprothrombin II when purified prothrombin is the substrate or when the prothrombin is in the original plasma or whole blood. Extraction of purified platelet factor 3 leaves a protein residue and it is believed that platelet factor 3 is a lipoprotein. The infrared absorption spectrum of the lipid obtained from purified platelet factor 3 shows strong absorption bands. From the general absorption pattern, we infer that the lipid is not a heterogeneous mixture, but most likely a single substance or consists of several that are nearly alike. Platelet factor 3 lipid(s) is most likely a cephalin-like compound.

## Introduction

There are numerous substances concerned with the activation of prothrombin. These can first be removed from prothrombin by purification, and then it is possible to observe activation with selected factors and biochemical preparations. Thromboplastin from lung tissue or brain and calcium ions yield some biothrombin slowly when added to purified prothrombin and the remainder of the prothrombin becomes a derivative that is not biothrombin (34). As early as 1939 it was found that biothrombin, when mixed with prothrombin preparations, destroys the traditional activity of prothrombin (19). We now know that "inactivation" of the prothrombin may actually consist of the formation of a prothrombin derivative of importance in the blood clotting mechanisms. Moreover, to obtain this derivative, it is necessary to have Ac-globulin as well as biothrombin. When observed by electrophoresis the "inactive" prothrombin has a lower mobility than prothrombin itself (31). It possesses the properties of a platelet cofactor called autoprothrombin II[2] which functions in the conversion of prothrombin to thrombin as follows:

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[2]*This derivative is apparently the same substance as platelet cofactor II (16), Plasma Thromboplastin Component (1), Christmas factor (7), or factor IX (18).*

$$\text{Prothrombin} \xrightarrow[\text{Ac-globulin}\ +\ \text{Platelet factor 3}\ +\ \text{Autoprothrombin II}]{Ca^{++}} \text{Biothrombin.} \qquad [1]$$

Another derivative, autoprothrombin I, is obtained from prothrombin with calcium ions, Ac-globulin, and platelet factor 3 (26). Its properties are quite different from autoprothrombin II. It promotes the conversion of prothrombin to biothrombin as follows:

$$\text{Prothrombin} \xrightarrow[\text{Ac-globulin}\ +\ \text{Thromboplastin}\ +\ \text{Autoprothrombin I}]{Ca^{++}} \text{Biothrombin.} \qquad [2]$$

It is now apparent that there are several ways to obtain biothrombin from prothrombin and several ways to obtain each of the other derivatives of prothrombin (25). In this paper we consider lipid materials obtained as crude extracts from brain and platelets as activators of prothrombin. Under suitable conditions these extracts are found to function in the production of biothrombin and under other conditions they fail to do so but still possess the property of activating prothrombin; however, the activation consists of the conversion of prothrombin to autoprothrombin II.

## Materials and Methods

*Prothrombin*

Purified prothrombin was prepared from bovine plasma by the methods of Seegers and associates (34, 30, 24). Whenever the final product contained Ac-globulin this activity *was removed* by heating as described by Ware and Seegers (34). The solutions usually were made up to contain 10,000 $\mu$/ml. as measured by two-stage assay (35).

*Purified Platelet Factor 3*

This was obtained from bovine platelets by the procedure described by Alkjaersig, Abe, and Seegers (2). The platelet factor 3 concentration was 500 $\mu$/ml. This preparation has not been exactly characterized in terms of chemical composition. Although no attempts have been made to show that it is a single substance, it is free of all other known blood coagulation factors. During the purification, a yield of 65% is obtained, and about 95% of the original phosphorus, nitrogen, and carbohydrate of the platelets is removed.

*Ether Extract of Platelet Factor 3*

Purified platelet factor 3 was sedimented at 106,000 *g* for two hours in a Spinco Ultracentrifuge. The sediment was extracted several times with ether. The ether extracts were kept dry with anhydrous sodium sulphate. The combined solutions from three extractions were placed in a tissue homogenizer (Potter). The ether was then evaporated with the aid of a stream of nitrogen

gas. Saline was then added to the lipid material to obtain an opalescent turbid suspension. The material obtained from 10 ml. of packed platelets was represented in a final volume of 6 ml.

*Ether Extract of Whole Platelets*

Platelet suspensions were dried with acetone. The dried platelets were then extracted with ether. These extracts were then dried and suspended in saline as described above. The final preparation contained the equivalent of 5 ml. of packed platelets per milliliter of lipid suspension.

*Ether Extract of Brain*

A cow brain was obtained from the packing plant and immediately washed free of blood. The membranes and white matter were roughly removed by dissection. The brain was then dried with the use of acetone. One gram of dried brain was extracted with 50 ml. of ether. The ether was evaporated and a saline suspension of the lipid material was obtained as described above. The material from 1 gm. of dried brain was suspended in 50 ml. of saline. This extract was found to be very active in the thromboplastin generation test and by that criterion retained its activity from two to three days at room temperature; however, it was ordinarily stored in a deep freeze.

*Thromboplastin Generation Test*

The method described by Biggs and Douglas (6) was modified as described by Duckert *et al.* (13).

*Analytical Methods*

Prothrombin concentration was measured by the modified two-stage procedure of Ware and Seegers (35), thrombin by the procedure of Seegers and Smith (32). Inactive derivative of prothrombin was determined by two-stage prothrombin analysis. The difference between the original prothrombin concentration and that found with a later two-stage analysis represented the amount of prothrombin derivative. That activity which could not be identified as thrombin or prothrombin was considered derivative. To determine whether autoprothrombin I or autoprothrombin II was formed the methods previously described for recognizing autoprothrombin I and autoprothrombin II activity were used (26, 28).

*Adsorbed Bovine Serum*

Ten milliliters of bovine serum were mixed with 500 mgm. $BaCO_3$. The mixture was centrifuged. The serum was stored in a deep freeze.

## Experimental

*Lipid of Platelet Factor 3*

Previous work on the purification (2) of platelet factor 3 indicated that this clotting factor is extensively purified by the methods devised. This made it attractive to study the lipid material derived from the preparation. Because of the uncertainty about associated impurities, this information would not

definitely describe the exact chemical nature of platelet factor 3 lipid. However, we were not likely to be dealing with samples that contained mainly extraneous material, and thus the probable chemical nature of platelet factor 3 lipid would be indicated from our studies. By extracting purified platelet factor 3 preparations with ether, a residue consisting of protein is obtained. The latter has not been studied. This finding, together with other properties, leads us to the view that platelet factor 3 is a lipoprotein. The ether solutions of the lipid are water clear and when the ether is evaporated the lipid is soluble in alcohol and chloroform but not in acetone. It contains most of the phosphorus of the original platelet factor 3 preparation. In qualitative tests, no choline was found. Tests for unsaturated bonds were all distinctly positive. The dry ether extract was light yellow in color and after evaporation of the ether it was easy to make soft smears. It was thus possible to obtain a thin film between salt crystals for measuring infrared absorption properties (Fig. 1).

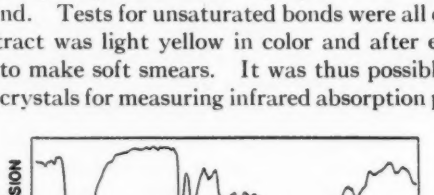


FIG. 1. Infrared absorption spectrum of platelet factor 3 lipid produced on a Beckman IR – 2T spectrophotometer. A weak absorption band was also found at 13.80. The base line represents zero and the top 100% transmission.

Strong absorption bands were found, suggesting one or a family of nearly similar substances rather than a grossly heterogeneous mixture. The various absorption bands correspond more to those of cephalin as published by Baer, Maurukas, and Russell (4) than any other we have seen. All of the properties we have found indicate that a cephalin-type compound comprises the lipid portion of platelet factor 3. This corresponds with the previous work of Chargaff, Bancroft, and Stanley-Brown (9), for they were able to isolate cephalin-like materials from platelets. Of the several lipid fractions they obtained, the cephalin fraction was most active in accelerating the clotting of plasma.

*Formation of Autoprothrombin II*

In previous work, it was found that autoprothrombin II is obtained from prothrombin by addition of a small amount of thrombin (19, 31, 28). This conversion occurred provided Ac-globulin was also in the reaction mixture. Calcium ions were found to inhibit the reaction (21). Since the conversion of prothrombin to thrombin can be achieved in many different ways, it seemed likely that the activation to autoprothrombin II might be facilitated in more than one way. We find that it is. Instead of using thrombin to initiate the activation of prothrombin, lipid materials can be used.

Activator substances could be obtained as ether extract of dry brain[3] (experiment 3, Table I), ether extract of whole platelets (experiment 6, Table I), or an ether extract of purified platelet factor 3 (experiment 9, Table I). We supplied the Ac-globulin in the form of adsorbed bovine serum. By heating the serum, the Ac-globulin was destroyed and autoprothrombin was not obtained (compare experiments 6 and 7 of Table I). In all of these experi-

TABLE I

YIELD OF REACTION PRODUCTS FOLLOWING PROTHROMBIN ACTIVATION

| Experiments* | Per cent yield after approximately three hours of activation | | | Remarks |
|---|---|---|---|---|
| | Prothrombin | Thrombin | Derivative | |
| 1. Prothrombin (2000 $\mu$/ml.) + $BaCO_3$ adsorbed serum | 100 | 0 | 0 | No activation |
| 2. Prothrombin (2000 $\mu$/ml.) + ether extract of brain | 100 | 0 | 0 | No significant activation. Some derivative can form in 24 hr. |
| 3. Prothrombin (2000 $\mu$/ml.) + ether extract of brain + $BaCO_3$ adsorbed serum | 12 | 2 | 86 | The derivative is autoprothrombin II. No autoprothrombin I |
| 4. Prothrombin (2000 $\mu$/ml.) + ether extract of brain + $BaCO_3$ serum (heated) | 88 | 2 | 10 | Yield of derivative is low even with very concentrated ether extracts of brain |
| 5. Prothrombin (2000 $\mu$/ml.) + ether extract of platelets | 94 | 0 | 6 | |
| 6. Prothrombin (2000 $\mu$/ml.) + ether extract of platelets + $BaCO_3$ adsorbed serum | 34 | 2 | 64 | The derivative is autoprothrombin II. No autoprothrombin I |
| 7. Prothrombin (2000 $\mu$/ml.) + ether extract of platelets + $BaCO_3$ adsorbed serum (heated) | 100 | 0 | 0 | |
| 8. Prothrombin (2000 $\mu$/ml.) + ether extract of platelet factor 3 | 100 | 0 | 0 | |
| 9. Prothrombin (2000 $\mu$/ml.) + ether extract of platelet factor 3 + $BaCO_3$ serum | 40 | 0.2 | 60 | The derivative is autoprothrombin II. No autoprothrombin I found |
| 10. Prothrombin (2000 $\mu$/ml.) + ether extract of platelet factor 3 + $BaCO_3$ serum (heated) | 100 | 0 | 0 | |
| 11. Prothrombin + platelet suspension | 62 | 3 | 35 | The derivative is autoprothrombin II |
| 12. Prothrombin (2000 $\mu$/ml.) + platelet suspension + calcium (0.027 *M*) | 35 | 20 | 45 | The derivative is autoprothrombin I. Calcium determines nature of derivative. Compare with experiment 11 |
| 13. Prothrombin (2000 $\mu$/ml.) + ether extract of platelets + $BaCO_3$ adsorbed serum + calcium (0.014 *M*) | 80 | 0 | 20 | Low calcium concentration and some autoprothrombin II is obtained |
| 14. Prothrombin (2000 $\mu$/ml.) + ether extract of platelets + $BaCO_3$ adsorbed serum + calcium (0.027 *M*) | 100 | 0 | 0 | High calcium concentration inhibits autoprothrombin II formation. Compare with experiment 6 |

**In each experiment four equal volumes of the reagents were mixed, and when a third or fourth one is not indicated saline was used. All concentrations refer to the final mixture. For example, in experiment 12 the following solutions were combined: 1 ml. prothrombin 8000 $\mu$/ml. plus 1 ml. platelet suspension plus 1 ml. $CaCl_2$ (0.108 M) plus 1 ml. saline.*

*[3]A refined product obtained from brain by Jensen and Therriault, Army Medical Research Laboratory, Fort Knox, Kentucky, was also studied. Their product was very active in the thromboplastin generation test and in the formation of autoprothrombin II, and inactive with calcium and Ac-globulin in the conversion of purified prothrombin to thrombin.*

ments, a small amount of thrombin was also obtained along with the autoprothrombin II. Lipid materials from several sources, including purified platelet factor 3, promote the activation of prothrombin to autoprothrombin II if Ac-globulin is supplied.

When thrombin and Ac-globulin are used to convert prothrombin to autoprothrombin II, calcium ions tend to inhibit the reaction. It was, therefore, likely that calcium would function in a similar manner when lipids are used to obtain autoprothrombin II. To measure the possible inhibitory effect of calcium ions in the activation of prothrombin to autoprothrombin II, several concentrations of calcium were studied (experiments 6, 13, and 14, Table I). With low concentrations, autoprothrombin II is obtained; when the concentration is higher than found in plasma, the reaction is virtually blocked (experiment 14, Table I).

It is not sufficient merely to inquire whether calcium ions inhibit or support the activation of prothrombin. Under certain conditions, *calcium determines the nature of the prothrombin derivative.* A mixture of prothrombin and platelet suspension yields autoprothrombin II; and the presence of calcium in 0.027 *M* concentration, in the reaction mixture, changes the reaction to yield autoprothrombin I (compare experiments 11 and 12, Table I). This determining role no longer applies when ether extracts of platelets are used in place of whole platelet suspensions or in place of purified platelet factor 3. Autoprothrombin I has never been obtained with simple ether extracts of platelets, or for that matter with ether extracts of brain. It seems likely that only autoprothrombin II can be obtained with the use of these lipid materials.

In the form of platelet factor 3, lipid is evidently combined with protein as lipoprotein and this material can be used to convert prothrombin to autoprothrombin I. The lipid alone promotes the formation of autoprothrombin II (experiment 9, Table I). The difference between lipid and platelet factor 3 is thus rather remarkable. Presumably, thromboplastin of brain or lung is also a lipoprotein that can be used to obtain biothrombin from prothrombin, but when the lipid material is first separated and used only autoprothrombin II can be obtained from prothrombin (experiment 3, Table I). The degradation product of brain thromboplastin preparations or of platelet factor 3 is sufficient to produce only a derivative of prothrombin but not sufficient to produce the biothrombin.

*Thromboplastin Generation Test*

A procedure devised by Biggs and Douglas (6) combines plasma, serum, and platelet factors, and thromboplastin generates over a period of approximately five minutes. The word "thromboplastin" is not used by Biggs and Douglas in the same sense which is customary for this laboratory. With this test, it was possible to take away the platelets and substitute either purified platelet factor 3, ether extract of platelet factor 3, ether extract of dried brain, or ether extract of platelets, and obtain "thromboplastin generation". Thus, in this test all of the lipid materials, irrespective of their source, possessed

TABLE II

ACTIVITY OF LIPID EXTRACTS OF BRAIN AND PLATELETS

| | Ether extract of platelet factor 3 | Ether extract of whole platelets | Ether extract of brain | Remarks |
|---|---|---|---|---|
| 1. Activity in formation of *Autoprothrombin II* from purified prothrombin | ++ | +++ | ++++ | The reaction occurs in plasma as well as with purified prothrombin; however, Ac-globulin is necessary in both cases and small amounts of thrombin form during the activation |
| 2. Activity in *Thromboplastin Generation Test* | ++ | +++ | ++++ | The extracts substitute for platelet factor 3 in this assay and produce an 11 to 14 second plasma clotting time after five minutes' incubation. The same incubation material as used in the Thromboplastin Generation Test will produce autoprothrombin II and small amounts of thrombin when added to purified prothrombin |
| 3. Activity in formation of *Thrombin** from purified prothrombin | – | – | – | The extracts do not substitute for platelet factor 3 in the formation of large amounts of thrombin and do not combine with any known substance to convert purified prothrombin to thrombin |

**Purified prothrombin was mixed with the extracts in combination with calcium, Ac-globulin, and diluted plasma; with calcium, Ac-globulin, and diluted serum; or with calcium, Ac-globulin, and autoprothrombin II. The experiments were designed in a manner similar to those previously described (29).*

coagulant activity (experiment 2, Table II). It was of interest to find that material from the thromboplastin generation test could be mixed with purified prothrombin to yield a small amount of thrombin; large quantities of autoprothrombin II and some prothrombin remained unactivated.

*Formation of Biothrombin*

Platelet factor 3 activity is measured in several ways: (*a*) with purified prothrombin, Ac-globulin, calcium ions, and autoprothrombin II, thrombin is obtained; (*b*) with purified prothrombin, calcium ions, Ac-globulin, and Linadryl, thrombin is obtained; and (*c*) with purified prothrombin, calcium ions, Ac-globulin, and platelet cofactor I, thrombin is also obtained. Platelet factor 3 has an important function in the activation of prothrombin to biothrombin in the above-sighted combinations. This function is *not* possessed by ether extracts of platelet factor 3. The complete platelet factor 3 is required; but, as has already been mentioned above, its lipid part is active in the formation of autoprothrombin II and in the thromboplastin generation test. Apparently the lipid is removed from a protein moiety, and the latter is of great importance (experiment 3, Table II).

In addition to working with the ether extracts of purified platelet factor 3 that are difficult to obtain we simply made an ether extract of brain and an ether extract of whole platelets as described above. It was not surprising to find that these extracts also do not yield much thrombin when mixed with purified prothrombin, calcium ions, and Ac-globulin (experiment 3, Table II). In fact this has previously been emphasized (29, 27). However, it was not

known what these lipid materials need to be combined with to function in the conversion of prothrombin to thrombin, namely, the residue left by the ether extraction.

*Autoprothrombin II Activity in Blood and Plasma*

The fact that the conversion of prothrombin to autoprothrombin II takes place in solutions of low calcium concentration and even in the absence of calcium (experiment 12, Table I) prompted us to see whether the prothrombin in ordinary citrated human plasma could be changed to autoprothrombin II by simply adding ether extract of brain. We found that about half the prothrombin activity could be made to disappear in two hours when large amounts of brain extract were used. This change was accompanied by a large increase of autoprothrombin II activity. Fresh blood was also added to ether extract of brain in the respective proportion of 10 to 1 and after two hours the serum was analyzed. It contained much more autoprothrombin II activity than is ordinarily found in serum. Consequently, the conversion of prothrombin to autoprothrombin II occurs in plasma and in clotting blood as well as with purified preparations of prothrombin. This opens the possibility of easily preparing autoprothrombin II for clinical use. Moreover, we may suppose that some autoprothrombin II can form in circulating blood.

The work of Chargaff describes thromboplastin obtained from lung extract as a macromolecular lipoprotein (10, 11). We may postulate that the ordinary thromboplastin of brain extract is also a lipoprotein protein. It is capable of activating prothrombin to thrombin under suitable conditions; namely, in association with calcium ions and Ac-globulin the interaction is supported or accelerated by autoprothrombin I (cf. equation 2). If thromboplastin is mixed with prothrombin and calcium ions in the absence of Ac-globulin, the yield of thrombin obtained is small (34). We now find that the remaining prothrombin is converted to autoprothrombin I. The following equation indicates the reaction:

$$\text{Prothrombin} \xrightarrow[\text{Thromboplastin}]{Ca^{++}} \text{Autoprothrombin I} + \text{Thrombin.} \qquad [3]$$

The lipid portion of the thromboplastin used in our experiments as ether extracts of brain does not function in the conversion of prothrombin to thrombin, but it retains the property of activating prothrombin to autoprothrombin II. Calcium is not required for this and may inhibit in suitable concentrations. The equation is as follows:

$$\text{Prothrombin} \xrightarrow[\text{Ac-globulin}]{\text{Lipid}} \text{Autoprothrombin II} + \text{Thrombin.} \qquad [4]$$

The thromboplastin obtained in crude brain preparations or crude lung extracts possesses several characteristics not manifested by any other known biochemical material. It has the following characteristics: (*a*) It can function with calcium, Ac-globulin, and autoprothrombin I in the conversion

of prothrombin to thrombin. (*b*) It can support the activation of prothrombin to autoprothrombin I in the presence of calcium ions. (*c*) When injected intravenously there is widespread intravascular coagulation. (*d*) A lipid fraction can be obtained from thromboplastin preparations that function with Ac-globulin to convert prothrombin to autoprothrombin II. The latter lipid fraction is quite active in the thromboplastin generation test. Presumably, the partial thromboplastin test of Brinkhous and associates (8) involves only the use of a part of the original thromboplastin and it seems to us that this observation also applies to the materials used in certain other studies (22, 5, 14). Indeed, the time-honored idea that cephalin or lipid is thromboplastin or is concerned with blood coagulation is supported, with, however, a reorientation in point of view. Thus, we can clearly see that the word "thromboplastin" refers to certain characteristics presumably possessed by a macromolecule. We might use the following equation:

$$\underset{\text{(Thromboplastin)}}{\text{Lipoprotein}} = \underset{\text{(Inert protein)}}{\text{Protein}} + \underset{\text{(Partial thromboplastin)}}{\text{Lipid}} . \quad [5]$$

No one seems to have succeeded in recombining the parts; however, Studer (cited in Reference 12) reports thromboplastin activity obtained from a combination of alcohol–ether extract of thromboplastin and the residue. In this connection, the speculations of Overman (20) are also of interest for he considered that the protein portion might possess inhibitor properties by itself.

It seems fairly certain from our work that platelet factor 3 is also a lipoprotein. In order to function in the transformation of prothrombin to thrombin, it requires calcium ions and Ac-globulin in a manner similar to thromboplastin; but then the analogy between the two stops. Platelet factor 3 requires a cofactor that can be supplied in the form of histamine, autoprothrombin II, or platelet cofactor I (cf. equation 1). The fact that histamine, autoprothrombin II, and platelet cofactor I are interchangeable in this reaction does not preclude the possibility that they are interchangeable in serving other functions. For example, platelet cofactor I can combine with an inhibitor during the clotting of blood (17). When the lipid portion is removed from the lipoprotein, the cofactors are not able to function in the conversion of prothrombin to thrombin. The lipid portion, however, is of value in the activation of prothrombin in that it promotes the transformation of prothrombin to autoprothrombin II, the latter being a cofactor of the original lipoprotein called platelet factor 3. When platelet factor 3 is injected intravenously extensive blood clotting does not follow presumably because Ac-globulin must first be converted to serum Ac-globulin before platelet factor 3 is effective with its cofactor.

For the multiple functions that are being ascribed to Ac-globulin, it is, in certain instances, not known whether plasma Ac-globulin can serve adequately or whether serum Ac-globulin is needed, and obtained according to the following equation:

$$\text{Plasma Ac-globulin} \xrightarrow{\text{Thrombin}} \text{Serum Ac-globulin.} \quad [6]$$

The conversion of prothrombin to biothrombin with platelet factor 3 and platelet cofactor I definitely requires serum Ac-globulin (17). That is also true when histamine replaces platelet cofactor I, and most likely true when autoprothrombin II replaces platelet cofactor I. By contrast there are indications that thromboplastin functions with both plasma or serum Ac-globulin. It also seems likely that plasma Ac-globulin supports the interactions which give autoprothrombin I and autoprothrombin II, and that it is not necessary to convert plasma Ac-globulin to serum Ac-globulin. However, these are all indications requiring further experimentation. At this time we can only call attention to the possibility that serum Ac-globulin is not the only form of this protein which functions in the blood clotting mechanisms.

Our work indicates that autoprothrombin II is most easily formed in solutions with low calcium concentration. The clotting of whole blood is known to be most effective at "optimum" calcium concentration. This refers to the total reactions taking place under the conditions of the observation. We still do not know the most favorable concentration for each of the chemical reactions in which calcium is concerned. Several specific functions of calcium are indicated by the following: interaction of thromboplastin and stable factor (autoprothrombin I) (15); conversion of prothrombin to autoprothrombin I (26); inactivation of platelet cofactor I (3); inactivation of thromboplastin (33); role in clot retraction; alteration in solubility characteristics of fibrin (23); acceleration of thrombin fibrinogen interaction (32); promotion of thrombin–thrombin formation (21); inhibition of autoprothrombin II formation (21); conversion of prothrombin to biothrombin. When all of these interactions occur in a single mixture, "optimum" calcium concentration is found and this consists of the most favorable average of all the reactions that are occurring. This average may not be the ideal calcium concentration for certain of the individual reactions. The quantitative calcium requirement for each reaction remains to be determined.

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# STUDIES OF FATTY ACID OXIDATION

## 4. THE EFFECTS OF FATTY ACIDS ON THE OXIDATION OF OTHER METABOLITES[1]

By P. G. Scholefield


### Abstract

Fatty acids inhibit the oxidation of pyruvate by rat-kidney mitochondria but the extent of inhibition depends upon the nature and amount of agent added to stimulate the oxidation. The longer chain fatty acids are more effective inhibitors and, in general, the even-numbered fatty acids show greater inhibitory effects than the adjacent odd-numbered fatty acids. Under conditions where 2,4-dinitrophenol (DNOP) and the fatty acids separately have little effect on the respiratory activity of rat-kidney mitochondria with pyruvate as substrate, the addition of both fatty acid and DNOP results in an extensive inhibition. At low concentrations the fatty acids are oxidized by rat-kidney mitochondria but at concentrations of $10^{-3}$ *M* and higher they inhibit their own oxidation, the oxidation of pyruvate, and those of the acids of the tricarboxylic acid cycle. The oxidation of pyruvate by rat-brain mitochondria is insensitive to decanoate but both the fumarate- and DNOP-stimulated oxidations of pyruvate are sensitive to the presence of decanoate. In contrast, Nembutal inhibits both the oxidation of pyruvate alone and the fumarate-stimulated oxidation of pyruvate. Possible mechanisms for the observed inhibitory effects of fatty acids are discussed.


### Introduction

The oxidation of fatty acids takes place readily in the presence of preparations of rat- or rabbit-liver mitochondria but with rabbit-kidney mitochondria (8) the oxidation is relatively slow. The exact conditions necessary for the oxidation of fatty acids by rat-kidney mitochondria do not seem to have been determined. On the other hand, in the presence of slices of kidney cortex from rat, rabbit, or guinea pig (14) and rabbit-kidney "cyclophorase" preparations (5), oxidation of fatty acids proceeds readily. With liver slices from the guinea pig, some inhibitory effects of fatty acids on respiratory activity and acetoacetate formation may be obtained on increasing the concentration of fatty acid above the optimal (14). The effect, however, is more obvious when rat-liver mitochondria are used instead of rat-liver slices (9). It has since been suggested by Lehninger (10) that this autoinhibition of the oxidation of fatty acids is due to an uncoupling of oxidation from phosphorylation by the fatty acids.

Uncoupling agents, such as 2,4-dinitrophenol (DNOP), have a characteristic effect on respiration which was first observed by Clowes and Krahl (2) in fertilized *Arbacia punctulata* eggs. At low concentrations ($10^{-5}$ molar (*M*)), DNOP was found to stimulate respiration but at higher concentrations an inhibition of respiration was found. Hotchkiss (6) showed that gramicidin had a similar effect on the respiration of *Staphylococcus aureus* and other bacteria. Further, both DNOP ($10^{-5}$ *M*) and gramicidin ($2.5 \times 10^{-7}$ *M*) were shown by Cross, Taggart, Covo, and Green (3) to stimulate the respiration of rabbit-kidney

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*Contribution from McGill–Montreal General Hospital Research Institute, 3619 University St., Montreal, Quebec.*

"cyclophorase" preparations by 20–35% in the presence of α-oxoglutarate. The concentrations of DNOP at which stimulation of respiration occurred were shown by Loomis and Lipmann (13) to prevent the aerobic synthesis of adenosine triphosphate (ATP). It was later claimed (7, 12) that the inhibition of pyruvate oxidation produced by the higher concentrations of DNOP could be reversed by addition of dicarboxylic acids of the tricarboxylic acid cycle. Similarly, Lehninger (9) had observed some reversal of the autoinhibition of octanoate oxidation by rat-liver mitochondria on addition of α-oxoglutarate.

It was of interest, therefore, to determine whether the aliphatic fatty acids could influence respiration of mitochondrial preparations in a manner analogous to that produced by uncoupling agents such as DNOP and gramicidin, and under what conditions any inhibition of respiration might be reversed by addition of dicarboxylic acids of the tricarboxylic acid cycle. In view of the limited ability of rat-kidney, and particularly rat-brain, mitochondria to oxidize fatty acids, it was felt that these systems would provide suitable preparations with which to study any inhibitory effects of fatty acids.

## Experimental

*Animals.*—All animals used were 200–250 gm. hooded rats of a local inbred strain.

*Chemicals.*—All common chemicals were of reagent grade and were used without further purification. The malic acid was the L-isomer (Nutritional Biochemicals Corporation), ampoules of cytochrome *c* containing 50 mgm./5 ml. were obtained from Wyeth Incorporated, and the sample of gramicidin (used as an aqueous suspension) was generously given by Dr. W. Johnson (Frank W. Horners Ltd., Montreal). Octoxyacetic acid was kindly synthesized by Dr. R. M. Greenway of this Institute by condensation of the sodium salt of octyl alcohol with chloroacetic ester.

The potassium salts of the fatty acids were made by neutralizing the fatty acids with saturated ethanolic KOH. The salts of the lower fatty acids crystallized out on cooling; those of the higher fatty acids were precipitated with ether or with acetone. The products were well washed with ether or acetone and dried in a vacuum desiccator. Solutions of the potassium salts were made neutral to phenol red before use. This generally involved the addition of not more than one drop of *N* HCl to each 10 ml. sample of 0.02 *M* solution.

*Mitochondria.*—Schneider's method (16), with modifications, was used for the isolation of liver and kidney mitochondria and that of Brody and Bain (1) for brain mitochondria. The tissues (10–15 gm. liver, 5–6 gm. brain or kidney) were homogenized in a Potter–Elvehjem type all-glass homogenizer in 9 ml. 0.025 *M* sucrose. For liver and kidney, the medium was made alkaline by addition of 0.25 ml. *N* KOH. For brain, only the cerebral hemispheres were used. The homogenate was then diluted with 0.25 *M* sucrose and centrifuged for 10 min. at 600 *g* (liver and kidney) or at 1500 *g*

(brain), calculated for the middle of the tube. A PR2 International Centrifuge with Multispeed attachment was used. The supernatant was centrifuged at maximum speed (20,000 *g* at the middle of the tube) for 10 min. and the sediment was washed twice by resuspension and centrifugation for further periods of 10 min. each at the same speed. Any material not well sedimented was poured off with the supernatant. The final supernatant was quite clear, indicating the absence of gross contamination with microsomes. The mitochondria were then resuspended in the required volume of 0.25 *M* sucrose so that 1 ml. suspension contained mitochondria from 1.4 gm. liver, 1 gm. brain, or 600 mgm. kidney; 0.7 ml. was used per vessel.

*Respiratory activity* was measured with the conventional Warburg apparatus at 37° in an atmosphere of air. The rate of uptake of oxygen was measured over the first 60 min. period with liver and kidney and 40 min. with brain. Experiments were repeated three times unless otherwise stated and mean values are quoted. In general, the individual values did not differ from the mean by more than 10%.

*The incubation medium*, essentially that described by Judah and Rees (8), contained 0.02 *M* sodium phosphate pH 7.4, 0.001 *M* potassium adenylate, 0.0067 *M* magnesium sulphate, cytochrome *c* (500 μgm./3 ml.), and 0.033 *M* potassium chloride. This is termed the basic medium and all values refer to final concentrations. Acids were added as their potassium salts and a corresponding reduction was made in the amount of added potassium chloride. The final volume in all cases was 3 ml.

## Results

### *The Effect of Fatty Acids on the Oxidation of Sodium Pyruvate by Rat-kidney Mitochondria*

Preliminary experiments indicated that the fatty acids had an effect on the oxidation of pyruvate by rat-kidney mitochondria but that the effect depended upon the nature of the agent used to stimulate oxidation of the sodium pyruvate. In the absence of such an agent, pyruvate was oxidized at a rate which was approximately 70% of that observed with bicarbonate or malate present. This oxidation was inhibited by 75% on addition of $1.67 \times 10^{-3}$ *M* octanoate but the bicarbonate-stimulated oxidation was inhibited by only 25% and the malate-stimulated oxidation was actually increased by 15% on addition of octanoate (Table I).

A virtually complete inhibition of pyruvate oxidation resulted when DNOP was present at a final concentration of $5 \times 10^{-5}$ *M* if no stimulating agent was present or if bicarbonate was present. Addition of $5 \times 10^{-5}$ *M* DNOP apparently had little effect on the rate of pyruvate oxidation in presence of malate (Table I). Closer examination, however, revealed that in the first 30 min. period there was an increase of about 10% in the rate of respiration and in the second 30 min. period the rate decreased so that an inhibition of 10% was obtained. Further incubation resulted in more significant diminutions in the respiratory activity. These results are in accord with those of

TABLE I

THE EFFECT OF DNOP AND OCTANOATE ON PYRUVATE OXIDATION BY RAT-KIDNEY MITOCHONDRIA

The basic medium and $6.7 \times 10^{-3}$ *M* sodium pyruvate were present in each vessel. In the absence of pyruvate, values of less than 20 μl. were obtained in all cases. All figures refer to μl. oxygen taken up in the 60 min. following thermal equilibration for 6 min.

| Additions | 'Priming' agent added | | |
|---|---|---|---|
| | Nil | $5 \times 10^{-3}$ *M* potassium bicarbonate | $6.7 \times 10^{-4}$ *M* potassium malate |
| Nil | 323 | 470 | 449 |
| $1.67 \times 10^{-3}$ *M* octanoate | 84 | 358 | 524 |
| $5 \times 10^{-5}$ *M* DNOP | 20 | 24 | 446 |
| $5 \times 10^{-5}$ *M* DNOP + $1.67 \times 10^{-3}$ *M* octanoate | 19 | 18 | 110 |

Lipmann and Kaplan (12) and Judah (7) on the apparent reversal of the inhibitory effect of DNOP by dicarboxylic acids with rat-liver mitochondria.

When, however, both DNOP ($5 \times 10^{-5}$ *M*) and octanoate ($1.67 \times 10^{-3}$ *M*) were added to rat-kidney mitochondria oxidizing pyruvate in the presence of small quantites of malate, the respiratory activity ceased after 30 min. The total amount of oxygen taken up in this period amounted to 70% of the theoretical value for complete oxidation of the 2 micromoles (μM.) malate present. When both agents were added together with bicarbonate or in the absence of a primer, the oxidation of pyruvate was virtually eliminated as it was when DNOP alone was added (Table I).

In view of the inhibitory effects obtained with octanoate, the effects on respiratory activity of all fatty acids from butyric to dodecanoic, were compared. The results obtained are given in Table II. It is apparent that, whilst the respiratory activity with pyruvate and malate as substrate was quite consistent, the activity with pyruvate and bicarbonate present was sometimes a little lower, and with pyruvate alone the respiratory activity was quite variable (cf. Table I). In general, the lower fatty acids had little effect on the oxidation of pyruvate alone but octanoic and higher fatty acids markedly inhibited this oxidation. A similar picture presented itself when bicarbonate was present. Some alternation was apparent, odd-numbered fatty acids being somewhat less effective inhibitors than the even-numbered fatty acids. Thus, in the presence or absence of either bicarbonate or malate, hexanoic acid affected pyruvate oxidation in a manner that was quantitatively similar to that observed with nonanoic acid and to an extent which was greater than that observed with either pentanoic or heptanoic acid. With the longer chain fatty acids (dodecanoic and higher) precipitates were obtained in the vessels and the inhibition was complete in all cases. With decanoic and undecanoic acids there was no obvious precipitation but there were significant inhibitions of pyruvate oxidation even in the presence of malate.

TABLE II

THE EFFECT OF FATTY ACIDS, DNOP, AND 'PRIMING' AGENT ON PYRUVATE OXIDATION BY RAT-KIDNEY MITOCHONDRIA

The basic medium and $6.7 \times 10^{-3}$ *M* sodium pyruvate were present throughout. Final concentrations of the added materials were: DNOP, $5 \times 10^{-5}$ *M*; potassium salts of fatty acids, $1.67 \times 10^{-3}$ *M*; potassium malate, $6.7 \times 10^{-4}$ *M*; and potassium bicarbonate, $5 \times 10^{-3}$ *M*. All figures in the table refer to μl. oxygen taken up in the 60 min. following thermal equilibration for 6 min.

| DNOP | − | − | − | − | − | − | + | + | + | + | + | + |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Malate | − | − | − | − | + | + | − | − | − | − | + | + |
| Bicarbonate | − | − | + | + | − | − | − | − | + | + | − | − |
| Fatty acid | − | + | − | + | − | + | − | + | − | + | − | + |
| Butyric | 95 | 76 | 267 | 211 | 340 | 341 | 8 | 7 | 16 | 17 | 217 | 188 |
| Pentanoic | 110 | 70 | 311 | 305 | 379 | 388 | 11 | 11 | 19 | 20 | 271 | 236 |
| Hexanoic | 101 | 84 | 330 | 242 | 377 | 401 | 8 | 11 | 21 | 19 | 297 | 206 |
| Heptanoic | 157 | 130 | 335 | 319 | 380 | 412 | 9 | 9 | 21 | 27 | 300 | 249 |
| Octanoic | 182 | 37 | 384 | 152 | 401 | 486 | 13 | 13 | 24 | 27 | 352 | 166 |
| Nonanoic | 107 | 57 | 368 | 296 | 385 | 469 | 11 | 9 | 21 | 27 | 318 | 164 |
| Decanoic* | 125 | 14 | 396 | 17 | 397 | 198 | 10 | 8 | 20 | 15 | 355 | 56 |
| Undecanoic* | 163 | 20 | 382 | 34 | 385 | 120 | 13 | 13 | 14 | 17 | 410 | 98 |
| Dodecanoic* | 163 | 21 | 382 | 32 | 385 | 38 | 13 | 20 | 14 | 22 | 410 | 49 |

**All results are the mean of two separate experiments except the last three results which refer to individual experiments.*

When $5 \times 10^{-5}$ *M* DNOP was added, respiration was apparent only in those vessels where malate was also present. Again, there was evidence that the even-numbered fatty acids are more effective inhibitors than the odd-numbered and the first fatty acid to produce 50% inhibition at this concentration (0.00167 *M*) in the presence of DNOP was octanoic acid. However, when inhibitions were obtained on addition of both DNOP and fatty acid, it was nearly always true that the initial respiratory activity was unaltered, the effect on respiratory activity arising from a complete inhibition after 10–30 min.

*Comparison of Decanoic Acid with DNOP and Gramicidin*

The inhibitory effects of decanoic acid on pyruvate oxidation by rat-kidney mitochondria in the presence or absence of bicarbonate or malate were similar to those obtained with DNOP. The effects of various concentrations of decanoic acid, DNOP, and gramicidin on this system were therefore examined. It may be seen from Fig. 1 that there is some degree of similarity between the effects of these three compounds. The oxidation of pyruvate alone was in all cases most sensitive and the addition of decanoic acid, DNOP, or gramicidin in the presence of malate resulted in a significant acceleration of respiration. However, there was a characteristic effect produced by each compound. Addition of decanoic acid at concentrations as low as $1.3 \times 10^{-4}$ *M* produced a significant inhibition of the rate of pyruvate oxidation by the rat-kidney mitochondria and possible stimulations when either bicarbonate or malate was present. Increase in the amount of added decanoic acid produced a stimulation in the presence of added malate but approximately 50% inhibition

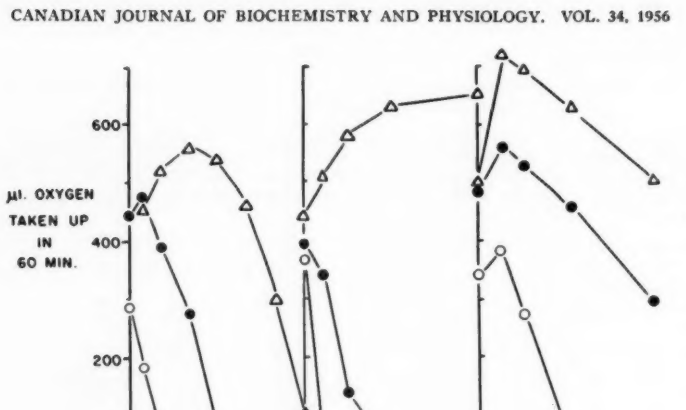


FIG. 1. The effects of decanoic acid, 2,4-dinitrophenol (DNOP), and gramicidin on the oxidation of $6.7 \times 10^{-3}$ *M* sodium pyruvate by rat-kidney mitochondria in the presence of various 'primers'. The prevailing conditions were as described in the 'Experimental' section. ○ No 'primer' added. ● $5 \times 10^{-3}$ *M* potassium bicarbonate added. △ $6.7 \times 10^{-4}$ *M* potassium L-malate added.

of the rate of oxygen uptake in the presence of added bicarbonate. In the presence of pyruvate alone there was a decrease in activity to a level which was not significantly higher than that found in the absence of substrate (pyruvate). Further increase in the decanoate concentration resulted in a marked decrease in the rate of pyruvate oxidation even in the presence of malate. On the other hand, within the limits of concentration examined ($5 \times 10^{-6} - 5 \times 10^{-5}$ *M*), addition of DNOP alone produced a stimulation of respiratory activity but no eventual inhibition when malate was present. Addition of gramicidin (2.5 µgm./ml.) produced some stimulation of pyruvate oxidation in all cases but at higher concentrations there was a gradually increasing inhibition.

Despite the similarity between the effect obtained with decanoic acid and that obtained with DNOP on pyruvate oxidation by rat-kidney mitochondria, it seemed possible that the apparent stimulations obtained with the fatty acid might rather be due to oxidation of the fatty acid. Further, since the major difference between the effects of decanoic acid and those due to DNOP lay in the extent of reversal of inhibitory action by malate, a more detailed examination of the nature of this reversal was made.

### *The Effect of Tricarboxylic Acid Cycle Intermediates on the Oxidation of Pyruvate in Presence of DNOP and Decanoate*

The results reported above (Table I and Fig. 1) confirmed those of Lipmann and Kaplan (12) and Judah (7) that the DNOP inhibition of pyruvate oxidation by rat-liver mitochondria can be reversed by the addition of intermediates

of the tricarboxylic acid cycle. The explanation offered by Judah (7) suggested that the oxidation of added malate supplied ATP even in the presence of DNOP. However, it seemed that an alternative explanation might be that the malate supplied additional oxaloacetate essential for the oxidation of pyruvate in mitochondrial systems. Accordingly, the effects of citrate, $\alpha$-oxoglutarate, fumarate, and malate on the oxidation of pyruvate by rat-liver mitochondria in the presence and absence of DNOP were investigated. The results obtained are given in Table III.

It is apparent that, at a concentration of $5 \times 10^{-5}$ *M*, DNOP markedly inhibits the oxidation of both pyruvate (mean value from 12 experiments, 47%) and the intermediates of the tricarboxylic acid cycle when the latter were added at the level of 2 $\mu$M./3 ml. ($6.7 \times 10^{-4}$ *M*). In the presence of these compounds the rate of oxidation of pyruvate was unchanged but the addition of DNOP to a mixture of pyruvate and any of these intermediates consistently resulted in a marked stimulation (57–83%) of respiratory activity.

When the concentrations of these intermediates were increased 10-fold to 20 $\mu$M./3 ml. ($6.7 \times 10^{-3}$ *M*), some variation was obtained on addition of DNOP. Thus, the oxidation of citrate was inhibited by 50%, that of malate not significantly altered, and the oxidation of fumarate and $\alpha$-oxoglutarate stimulated. Nevertheless, in all cases the oxidative activity in the presence of both pyruvate and the tricarboxylic acid cycle intermediates at this concentration was still markedly stimulated on addition of DNOP. In view of the lack of uniformity in the effect of $5 \times 10^{-5}$ *M* DNOP on the oxidation of $6.7 \times 10^{-3}$ *M* citrate, $\alpha$-oxoglutarate, fumarate, and malate, an attempt was made to estimate the concentrations of DNOP required to give maximal

TABLE III

THE INHIBITORY EFFECT OF DNOP ON PYRUVATE OXIDATION BY RAT-LIVER MITOCHONDRIA AND ITS REVERSAL BY VARIOUS CARBOXYLIC ACIDS

The basic medium was present throughout and $6.7 \times 10^{-3}$ *M* sodium pyruvate as indicated. All figures in this table are referred to an arbitrary standard of 100, set for the rate of oxygen uptake obtained on addition of pyruvate alone. This rate was the most consistent and the actual value was approximately 250 $\mu$l./60 min. period. All values in the case of each carboxylic acid were determined simultaneously and the mean value from three separate determinations is reported.

| Carboxylic acid | $5 \times 10^{-5}$ *M* DNOP | Additions | | | | | |
|---|---|---|---|---|---|---|---|
| | | Nil | Pyruvate | 2 $\mu$M. acid | 2 $\mu$M. acid + pyruvate | 20 $\mu$M. acid | 20 $\mu$M. acid + pyruvate |
| L-Malate | – | 24 | 100 | 47 | 91 | 74 | 98 |
| | + | 7 | 47 | 28 | 187 | 78 | 357 |
| Citrate | – | 19 | 100 | 63 | 104 | 119 | 135 |
| | + | 5 | 49 | 51 | 163 | 58 | 156 |
| $\alpha$-Oxoglutarate | – | 24 | 100 | 45 | 109 | 105 | 122 |
| | + | 6 | 65 | 39 | 182 | 198 | 279 |
| Fumarate | – | 26 | 100 | 44 | 104 | 85 | 122 |
| | + | 7 | 51 | 35 | 157 | 116 | 264 |

TABLE IV

THE CONCENTRATIONS OF DNOP REQUIRED TO PRODUCE MAXIMUM STIMULATION OF THE RATE OF OXIDATION OF VARIOUS CARBOXYLIC ACIDS BY RAT-LIVER MITOCHONDRIA

All substrates were present at a final concentration of $6.7 \times 10^{-3}$ *M* in the basic medium. The concentrations of DNOP examined were in the range $5 \times 10^{-6}$ *M*–$15 \times 10^{-5}$ *M*.

| Substrate | Concentration of DNOP to produce maximum stimulation |
|---|---|
| Nil | $5 \times 10^{-6}$ *M* |
| Citrate | $5 \times 10^{-6}$ *M* |
| Pyruvate | $10^{-5}$ *M* |
| Fumarate | $1.5 \times 10^{-5}$ *M* |
| Malate | $1.5 \times 10^{-5}$ *M* |
| α-Oxoglutarate | $3 \times 10^{-5}$ *M* |

stimulation with each of these substrates, using rat-liver mitochondria. It is apparent (Table IV) that this concentration of DNOP depends on the substrate.

When the effects of decanoic acid and DNOP on pyruvate oxidation by rat-kidney mitochondria in the presence of various quantities of fumarate were examined, the results given in Table V were obtained. The rate of oxidation of pyruvate by rat-kidney mitochondria was increased by approximately 15% on addition of fumarate, and the stimulation was constant, within experimental error, whether 0.6 μM. or 20 μM. of fumarate was added. Addition of decanoic acid or DNOP in the absence of fumarate resulted in inhibitions of pyruvate oxidation similar to those reported in Tables I and II

TABLE V

THE EFFECTS OF DECANOIC ACID AND DNOP ON PYRUVATE OXIDATION BY RAT-KIDNEY MITOCHONDRIA IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF FUMARATE

The basic medium and $6.7 \times 10^{-3}$ *M* sodium pyruvate were present throughout and further additions were as indicated. All values in each table were determined simultaneously and represent the mean value of three such determinations in μl. oxygen taken up in the 60 min. following thermal equilibration.

| | μM. fumarate added/3 ml. | | | | |
|---|---|---|---|---|---|
| | 20 | 6 | 2 | 0.6 | 0 |
| μM. decanoic acid added/3 ml. | | | | | |
| 0 | 476 | 483 | 448 | 462 | 402 |
| 2 | 651 | 675 | 691 | 679 | — |
| 4 | 723 | 818 | 772 | 759 | — |
| 6 ($2 \times 10^{-3}$ *M*) | 281 | 316 | 270 | 180 | — |
| Conc. of DNOP | | | | | |
| 0 | 505 | — | — | — | 433 |
| $1.5 \times 10^{-5}$ *M* | 710 | 695 | 645 | 612 | 50 |
| $5 \times 10^{-5}$ *M* | 805 | 808 | 611 | 172 | 19 |
| $15 \times 10^{-5}$ *M* | 449 | 437 | 159 | 63 | 18 |

and in Fig. 1. Addition of decanoic acid in the presence of fumarate resulted in increased respiratory activity when 2 or 4 $\mu$M. decanoic acid was added but decreased activity when 6 $\mu$M. was added; the activity was independent of the actual amount of fumarate present (0.6–20 $\mu$M./vessel) except when the highest concentration of fatty acid and the lowest concentration of fumarate were present. On the other hand, the effect of DNOP depended on the amount of fumarate present if it was less than 6 $\mu$M./3 ml. ($2 \times 10^{-3}$ *M*). When rat-liver mitochondria were substituted for rat-kidney mitochondria an essentially similar result was obtained.

A control set of experiments was run in the absence of added pyruvate (Table VI). The amount of oxygen taken up in the presence of 0.6 and 2 $\mu$M. of fumarate corresponded with complete oxidation of the substrate to $CO_2$ and water. Similar complete oxidations of the larger amounts of fumarate occurred if the experiment was allowed to proceed for more than 60 min. Addition of $6.7 \times 10^{-4}$ *M* decanoate resulted in an increase in respiratory activity. This increase could not be accounted for by complete oxidation of fumarate as insufficient fumarate was present. Thus, at this concentration, decanoic may be oxidized by rat-kidney mitochrondria. On addition of $1.67 \times 10^{-3}$ *M* decanoic acid, no significant respiratory activity was obtained unless at least 6 $\mu$M. ($2 \times 10^{-3}$ *M*) fumarate were present. Decanoate oxidation in presence of this and larger amounts of fumarate seems likely. In the presence of $2 \times 10^{-3}$ *M* decanoate, there was little respiratory activity; even the oxidation of fumarate itself was inhibited.

The results summarized in Tables V and VI would therefore tend to support the hypothesis that decanoate does not stimulate respiration of rat-kidney mitochondria but rather that, at low concentrations, it may itself be oxidized if fumarate or other dicarboxylic acid is present in sufficient quantity.

TABLE VI

THE EFFECTS OF DECANOIC ACID AND DNOP ON FUMARATE OXIDATION BY RAT-KIDNEY MITOCHONDRIA

Conditions as in Table V with the exception that no pyruvate was present.

| | $\mu$M. fumarate added/3 ml. | | | | |
|---|---|---|---|---|---|
| | 20 | 6 | 2 | 0.6 | Nil |
| $\mu$M. decanoic acid added/3 ml. | | | | | |
| 0 | 347 | 265 | 151 | 61 | 7 |
| 2 | 519 | 487 | 450 | 273 | — |
| 4 | 532 | 344 | 53 | 16 | — |
| 6 ($2 \times 10^{-3}$ *M*) | 77 | 57 | 38 | 22 | — |
| Conc. of DNOP | | | | | |
| 0 | 416 | 351 | 159 | 61 | 5 |
| $1.5 \times 10^{-5}$ *M* | 366 | 203 | 38 | 17 | — |
| $5 \times 10^{-5}$ *M* | 71 | 55 | 34 | 19 | — |
| $15 \times 10^{-5}$ *M* | 81 | 48 | 33 | 13 | — |

*The Effect of Decanoate and DNOP on the Respiratory Activity of Rat-brain Mitochondria*

Fatty acids are very slowly oxidized by brain (4, 19). A comparison was therefore made of the effects of DNOP and decanoate on the oxidation of various substrates by rat-brain mitochondria. The most significant effects were obtained with pyruvate ($6.7 \times 10^{-3}$ *M*) as substrate in the presence and absence of fumarate ($6.7 \times 10^{-4}$ *M*).

Decanoic acid, at the concentrations used, was apparently without effect on the oxidation of pyruvate, but the addition of fumarate ($6.7 \times 10^{-4}$ *M*) approximately doubled the rate of uptake of oxygen with pyruvate as substrate and this increased respiratory activity was particularly sensitive to the presence of decanoic acid. The increased respiratory activity obtained on addition of fumarate was 50% inhibited by approximately $7 \times 10^{-4}$ *M* decanoate (Fig. 2*a*). The endogenous activity of rat-brain mitochondria and the activity observed on addition of fumarate were not greatly affected by decanoic acid.

The effect obtained on addition of DNOP to these same systems is shown in Fig. 2*b*. There was little, if any, effect on the endogenous activity or on that obtained when $6.7 \times 10^{-4}$ *M* fumarate was added. However, addition of DNOP to a system oxidizing pyruvate resulted in very marked stimulations of respiratory activity. The addition of $6.7 \times 10^{-4}$ *M* fumarate again approximately doubled the activity in the absence of DNOP but the increase above that obtained with pyruvate alone diminished with increasing DNOP concentrations. Thus, whilst addition of either decanoate or of DNOP resulted in a marked decrease in the fumarate-stimulated oxidation of pyruvate only DNOP had an effect on the oxidation of pyruvate which is not fumarate-dependent.

When the complementary effects of decanoate and DNOP on pyruvate oxidation were examined, the results shown in Table VII were obtained. The concentration of DNOP used ($5 \times 10^{-5}$ *M*) was that which gave the greatest stimulation of pyruvate oxidation (Fig. 2*b*) and the concentration of decanoate was that which from Fig. 2*a* should give approximately 90% inhibition of the fumarate-stimulated pyruvate oxidation.

DNOP, decanoate, or these two compounds together, did not affect the endogenous activity or the activity obtained on addition of fumarate alone (Table VII). The effects of DNOP and decanoate separately on pyruvate oxidation in the presence and absence of fumarate were confirmed but on addition of DNOP and decanoate together the stimulation observed with DNOP alone was completely abolished in the presence or absence of $6.7 \times 10^{-4}$ *M* fumarate.

The effect of various concentrations of decanoate on the oxidation of pyruvate, with and without fumarate and/or DNOP present, was examined. The results obtained are shown in Fig. 3*a*. Both the fumarate and the DNOP-stimulated oxidations of pyruvate seem to be equally sensitive to decanoate. However, it seemed possible from these results that in the

presence of pyruvate there was some slight oxidation of the fatty acid itself. In order to eliminate this possibility, octoxyacetic acid ($C_8H_{17}OCH_2CO_2H$) was synthesized and its effect on these systems was investigated. This compound has the same carbon chain length as decanoic acid and preliminary unpublished experiments indicated that the oxyacetic acids are neither appreciably oxidized by rat tissues nor do they form coenzyme-A derivatives. The results obtained with octoxyacetate are shown in Fig. 3*b*. In this case

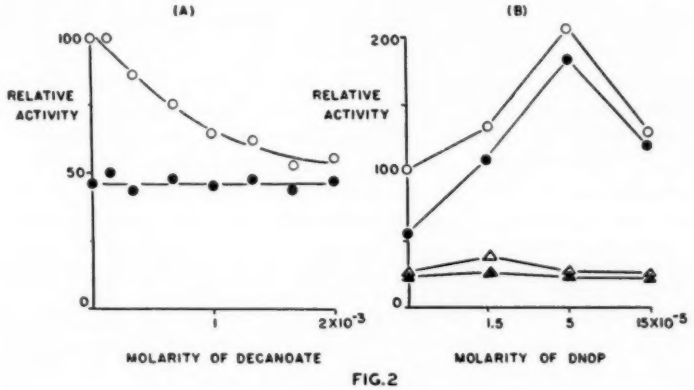


FIG. 2

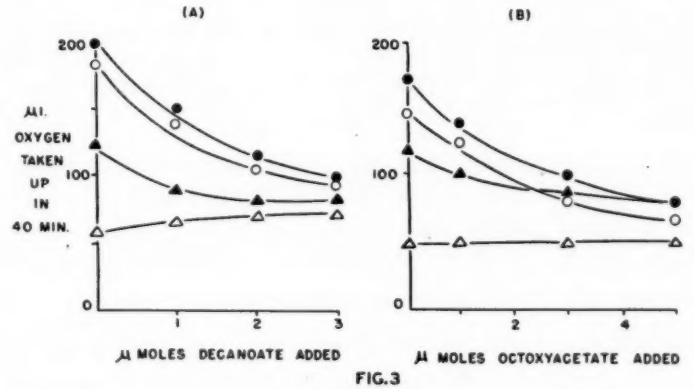


FIG. 3

FIG. 2. The effects of decanoic acid and 2,4-dinitrophenol (DNOP) on the oxidation of sodium pyruvate by rat-brain mitochondria in the presence and absence of fumarate. For purposes of comparison the rate of oxidation of pyruvate in the presence of fumarate has been arbitrarily set at 100. Conditions as described in the 'Experimental' section. ▲ No additions. △ $6.7 \times 10^{-4}$ *M* potassium fumarate added. ● $6.7 \times 10^{-3}$ *M* sodium pyruvate added. ○ $6.7 \times 10^{-4}$ *M* potassium fumarate and $6.7 \times 10^{-3}$ *M* sodium pyruvate added.

FIG. 3. The influence of decanoic and octoxyacetic acids on the oxidation of pyruvate by rat-brain mitochondria in the presence and absence of fumarate and/or 2,4-dinitrophenol (DNOP). $6.7 \times 10^{-3}$ *M* pyruvate was present throughout, other conditions as described in the 'Experimental' section. △ No further additions. ▲ $6.7 \times 10^{-4}$ *M* potassium fumarate added. ○ $5 \times 10^{-5}$ *M* DNOP added. ● $6.7 \times 10^{-4}$ potassium fumarate and $5 \times 10^{-5}$ *M* DNOP added.

TABLE VII

THE COMPLEMENTARY EFFECTS OF DECANOIC ACID AND DNOP ON PYRUVATE OXIDATION BY RAT-BRAIN MITOCHONDRIA

The basic medium was present throughout, other additions being as indicated. The figures quoted refer to μl. oxygen taken up in the 40 min. following thermal equilibration.

| Additions | Substrate | | | |
|---|---|---|---|---|
| | Nil | $6.7 \times 10^{-3}$ *M* pyruvate | $6.7 \times 10^{-4}$ *M* fumarate | $6.7 \times 10^{-3}$ *M* pyruvate + $6.7 \times 10^{-4}$ *M* fumarate |
| Nil | 33 | 74 | 46 | 156 |
| $1.67 \times 10^{-3}$ *M* potassium decanoate | 44 | 85 | 50 | 96 |
| $5 \times 10^{-5}$ *M* DNOP | 38 | 200 | 48 | 233 |
| $5 \times 10^{-5}$ *M* DNOP + $1.67 \times 10^{-3}$ *M* potassium decanoate | 40 | 87 | 44 | 100 |

there was definitely no oxidation of the fatty acid analogue but there was still an inhibitory pattern similar to that produced by decanoic acid.

A similar series of experiments with α-oxoglutarate, citrate, and malate as substrates revealed that all three substrates are oxidized by brain mitochondria α-oxoglutarate being oxidized most readily and citrate least. In no case was there any stimulation by DNOP, decanoate, or octoxyacetate. In all cases gradually increasing inhibitions were obtained and the sensitivity of these oxidations to DNOP, decanoate, and octoxyacetate seemed to be approximately the same as the sensitivity of the fumarate-stimulated pyruvate oxidation.

*The Effect of Nembutal on the Oxidation of Pyruvate by Rat-brain Mitochondria*

In view of its lipid nature and its characteristic effect on pyruvate oxidation in brain, it seemed possible that decanoic acid might be acting in a manner similar to that of certain narcotics. Several experiments were therefore

TABLE VIII

THE EFFECT OF NEMBUTAL ON PYRUVATE OXIDATION BY RAT-BRAIN MITOCHONDRIA

Conditions as in Table VII.

| Nembutal added | Substrate | | | |
|---|---|---|---|---|
| | Nil | $6.7 \times 10^{-3}$ *M* pyruvate | $6.7 \times 10^{-4}$ *M* fumarate | $6.7 \times 10^{-3}$ *M* pyruvate + $6.7 \times 10^{-4}$ *M* fumarate |
| Nil | 25 | 65 | 38 | 128 |
| $3.3 \times 10^{-4}$ *M* | 24 | 55 | 37 | 97 |
| $6.7 \times 10^{-4}$ *M* | 24 | 44 | 33 | 62 |
| $2 \times 10^{-3}$ *M* | 17 | 27 | 27 | 37 |

performed to determine the nature of the inhibition of pyruvate oxidation in rat-brain mitochondria by narcotics. Results obtained with Nembutal are given in Table VIII. It is apparent from these results that both the oxidation of pyruvate per se and the fumarate-stimulated oxidation of pyruvate are sensitive to Nembutal although the inhibitory effect on the latter is somewhat greater.

## Discussion

The inhibitory effects of fatty acids on their own oxidation by preparations of liver mitochondria are well known (see Ref. 9). It is now shown that this effect is not confined to the oxidation of fatty acids but that the oxidation of other substrates, particularly pyruvate, may be markedly inhibited by fatty acids. Rat-kidney or rat-brain mitochondria were used for these studies since they oxidize fatty acids to a very limited extent. For purposes of comparison, rat-liver mitochondria were sometimes used. In view of the suggestion by Lehninger (10) that the autoinhibitory effects of fatty acids are due to an uncoupling of oxidation from phosphorylation, most of the observed effects have been compared with those obtained on addition of DNOP.

The oxidation of pyruvate by rat-kidney mitochondria was inhibited by $1.67 \times 10^{-3}$ *M* octanoic acid but the effect depended upon the nature of the agent used to stimulate pyruvate oxidation in this system. The effects obtained on addition of $5 \times 10^{-5}$ *M* DNOP were similar to those produced by octanoate. The possibility arose, however, that the effects observed with octanoate were due to a combination of a simple inhibitory effect on some stage of pyruvate oxidation and an oxidation of octanoate by this preparation. Since it is well known that DNOP prevents the oxidative synthesis of ATP and that for the initial stage in fatty acid oxidation (the formation of a coenzyme-A derivative) ATP is essential, the effect of octanoate and DNOP together on pyruvate oxidation was investigated. In the presence of malate, neither octanoate nor DNOP alone showed any tendency to produce a marked inhibition whereas the addition of both DNOP and octanoate to rat-kidney mitochondria oxidizing pyruvate resulted in a 75% inhibition of respiratory activity. The observed effect of octanoate alone is probably due, therefore, to an inhibition of some respiratory system plus an increase in respiratory activity due to oxidation of the fatty acid itself. In the presence of DNOP the oxidation of octanoate is abolished and the inhibitory effect of the octanoate becomes apparent.

The influence of the priming agent on the effect of decanoate and the uncoupling agents on mitochondrial oxidation of pyruvate warrants some comment. Judah (7) suggested that the oxidation of the dicarboxylic acids supplied sufficient ATP, even in the presence of DNOP, to stimulate pyruvate oxidation. It seems more reasonable to suppose that the main function of these acids is to supply the oxaloacetate essential for the formation of citrate. Apparently pyruvate oxidation does not proceed in liver or kidney mitochondria without such formation of citrate (7). Bicarbonate also protects

against the inhibitory effects of DNOP in these systems (Fig. 1) and the recent demonstration (17, 18) that inosine triphosphate (ITP) is essential for the incorporation of $CO_2$ into pyruvate to form oxaloacetate offers support for the present hypothesis. It seems likely that ITP may be synthesized from the monophosphate and ATP (see Ref. 11) so that a decrease in ATP synthesis produced by DNOP may lead to a decreased synthesis of ITP and hence of oxaloacetate. Under these conditions, addition of oxaloacetate or compounds leading to its formation would be expected to reverse the inhibitory effect of DNOP.

Decanoate differed from DNOP in producing an inhibitory effect on pyruvate oxidation by rat-kidney mitochondria in the presence of malate. At low concentrations, both substances produced stimulations of respiration but whereas higher concentrations of DNOP produced no further effect, the addition of higher concentrations of decanoate began to result in decreased respiratory activity. Again it seems that the most reasonable explanation is that at low concentrations fatty acids are oxidized by rat-kidney mitochondria but at higher concentrations ($>10^{-3}$ $M$ in the case of decanoate) they begin to inhibit both their own oxidation and that of pyruvate.

The results quoted in Table II indicated that, in the presence of pyruvate with or without 'sparking' agent, there was little increased rate of oxygen uptake on the addition of fatty acids. An investigation was therefore made of the conditions under which decanoate is oxidized by rat-kidney mitochondria. In no case was any oxidation of the fatty acid observed in the absence of a priming agent or in the presence of bicarbonate. When fumarate was added, decanoate oxidation occurred as shown by the total oxygen uptake, but the rate depended upon the fumarate concentration and the decanoate concentration (Tables V and VI). When the concentration of decanoate was raised to $2 \times 10^{-3}$ $M$, little oxidation occurred whatever the concentration of fumarate present; even the rate of oxidation of fumarate was inhibited.

In order to obviate fatty acid oxidation as a factor in the inhibitory effect of decanoate on respiratory activity, rat-brain mitochondria were used in place of rat-liver or rat-kidney mitochondria. In this preparation pyruvate oxidation to acetate may occur and synthesis of citrate is not an essential corollary of pyruvate oxidation as in similar rat-liver and rat-kidney preparations. By adding or withholding fumarate, it is therefore possible to study the oxidation of pyruvate per se or the oxidation stimulated by fumarate, namely, that proceeding via the operation of the tricarboxylic acid cycle. In this way it was shown that decanoate has no effect on the pyruvic oxidase system producing acetate from pyruvate but that the further oxidation of pyruvate is quite sensitive to the presence of decanoate. It is of interest to note that Brody and Bain (1) have referred to an inhibitory effect of octanoate on the oxidation of small quantities of malate used for the stimulation of respiration of brain mitochondria.

Confirmation of the present results on the inhibitory action of fatty acids is forthcoming from those reported by Samson and Dahl (15). These authors

showed that intravenous injection of fatty acids (1 μM./gm. body weight in the rat) resulted in the production of a temporary comatose condition. A narcotic-like effect of the fatty acids would account for this result but when the effects of Nembutal and decanoate on pyruvate oxidation by rat-brain mitochondria were compared, certain differences were observed. For example, decanoate inhibits the fumarate-stimulated oxidation of pyruvate but not the oxidation of pyruvate alone, whereas Nembutal inhibits both oxidations, the fumarate-stimulated respiration being rather more sensitive.

The lack of effect of decanoate on the oxidation of pyruvate by rat-brain mitochondria and the ubiquity of the inhibitory effects on the complete oxidations of pyruvate and the intermediates of the tricarboxylic acid cycle make it unlikely that the pyruvic oxidase system is the site of the inhibition; it seems more likely that a phosphorylation reaction associated with these oxidations is primarily affected.

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# STUDIES OF FATTY ACID OXIDATION

## 5. THE EFFECT OF DECANOIC ACID ON OXIDATIVE PHOSPHORYLATION[1]

By P. G. Scholefield


### Abstract

The effects of potassium decanoate on the phosphorylation associated with the oxidation of pyruvate by rat-kidney and rat-brain mitochondria have been investigated. The suggestion that these two processes may be uncoupled from each other in the presence of decanoate has been confirmed. Further, it has been shown that the decanoate-insensitive oxidation of pyruvate by rat-brain mitochondria, occurring in the absence of such stimulating agents as fumarate, is not associated with ATP synthesis. The fumarate-stimulated oxidation of pyruvate by rat-brain mitochondria, which is inhibited by decanoate, is associated with a phosphorylation process which is uncoupled by decanoate. When pyruvate oxidation by rat-kidney or by rat-brain mitochondria is uncoupled from phosphorylation, the extent of uncoupling is proportional to the amount of decanoate added.


In the preceding paper (13) the effects of fatty acids on the respiratory activity of rat-kidney and rat-brain mitochondria with pyruvate as substrate were described. The ubiquity of the inhibitory effects indicated that a specific effect on a respiratory enzyme was unlikely. On the other hand a comparison of the general effects of fatty acids and 2,4-dinitrophenol (DNOP) on mitochondrial respiration gave little support to the suggestion of Lehninger (9) that fatty acids might act as uncoupling agents. The ability of fatty acids to uncouple the process of oxidation from that of phosphorylation was therefore determined directly.

### Experimental

In general, the conditions used were those described previously (13) except that a slight modification was made in the method of preparation of the mitochondria. In all cases the mitochondria were thrown down at 11,000 g (calculated for the middle of the tube) rather than at 20,000 g. Under this condition one washing with isotonic sucrose was apparently sufficient to remove most of the microsomes. In the preliminary experiments the temperature was maintained at 25° but later experiments indicated that at 37° the process of oxidative phosphorylation was nearly as efficient.

The general procedure used to measure oxidative phosphorylation was that described by Hunter (7). The medium contained 0.01 molar (*M*) sodium phosphate buffer pH 7.4, 0.001 *M* potassium adenylate, 0.0067 *M* magnesium sulphate cytochrome *c* (500 μgm./3 ml.), 0.033 *M* potassium chloride, and 0.01 *M* sodium fluoride, all values referring to the final concentration in the medium. Substrates and inhibitors were added as indicated in the text. After thermal equilibration 0.2 ml. 10 *N* $H_2SO_4$ or 0.2 ml. 0.3 *M* glucose containing 1 mgm.

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*Contribution from McGill–Montreal General Hospital Research Institute, 3619 University St., Montreal, Quebec. This work was supported by funds from the National Research Council of Canada.*

crystalline Sigma hexokinase was tipped into duplicates of the various Warburg flasks. The differences between the final phosphate levels in these pairs of flasks were a measure of the amount of phosphate taken up during the experimental period. The rates of uptake of oxygen were then determined and after 10 min. (kidney) or 25 min. (brain) the flasks were quickly removed, placed in cracked ice, and 1 ml. 15% trichloroacetic acid added. After centrifugation, phosphate determinations were made on 0.2 ml. aliquots of the supernatants using the method of Fiske and Subbarow (5).

## Results

*The Effect of Temperature on the Uncoupling Action of Decanoate*

All the preliminary experiments were conducted at 25° since, at this temperature, the P : O ratio is supposed to be maintained at its initial value for a longer period than at 37° (see, for example, Ref. 2). In these preliminary experiments on the oxidation of pyruvate by rat-kidney mitochondria evidence was obtained (Table I) that under the experimental conditions the addition

TABLE I

THE DECREASE IN P : O RATIO OBSERVED WHEN DECANOATE IS ADDED TO RAT-KIDNEY MITOCHONDRIA IN PRESENCE OF PYRUVATE ($6.7 \times 10^{-3}$ *M*) AND FUMARATE ($6.7 \times 10^{-4}$ *M*) AS SUBSTRATE AT TEMPERATURES OF 25° AND 37° C.

| | Decanoate added | | |
|---|---|---|---|
| *T* | 0 | $6.7 \times 10^{-4}$ *M* (2 μM./3 ml.) | $1.33 \times 10^{-3}$ *M* (4 μM./3 ml.) |
| 25° | 2.45* (100) | 1.35 (69) | 0.30 (44) |
| 37° | 2.17 (100) | 0.71 (65) | — — |

**The P : O values quoted for T = 25° are the means from six determinations; those for T = 37° are the means from four determinations. The values in parentheses refer to the relative respiratory activities, taking the control values as 100% in both series.*

of 2 micromoles (μM.) of potassium decanoate/3 ml. vessel contents ($0.67 \times 10^{-3}$ *M*) resulted in a 30% inhibition of respiration and 45% decrease in the P : O ratio. An increase in the amount of added decanoate to 4 μM./vessel resulted in corresponding decreases in both respiratory activity and in the P : O ratio, the extent of the decreases being apparently proportional to the amount of decanoate added. In order to compare the effects on respiration with those reported in the previous paper (13) the experiments were repeated at 37°. There was a drop of approximately 10% in the P : O ratio and the extent of uncoupling produced by $0.67 \times 10^{-3}$ *M* decanoate changed from a fall in P : O ratio of 1.10 at 25° to a fall of 1.46 at 37°. In view of the similarity between these two sets of results the more usual temperature of 37° was employed in all other experiments reported here.

*The Effect of Concentration of Decanoate on the Extent of Uncoupling*

An indication was obtained from the results of the experiments conducted at 25° that the extent of uncoupling produced by decanoate was directly proportional to the amount of decanoate present. This suggestion was verified by examination of the effects of several different concentrations of decanoate on the oxidation of pyruvate by rat-kidney mitochondria at 37°. The results obtained are shown in Fig. 1.

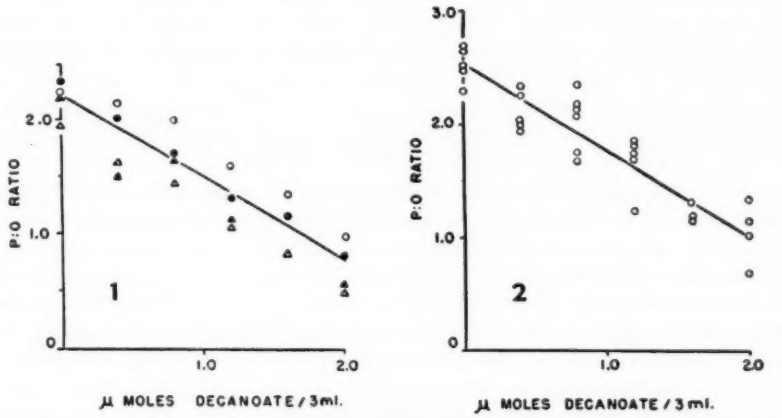


FIG. 1. The effect of decanoate on the P : O ratio obtained for the oxidation of $6.7 \times 10^{-3}$ *M* pyruvate and $6.7 \times 10^{-4}$ *M* fumarate by rat-kidney mitochondria. The various symbols refer to the results obtained in four separate experiments.

FIG. 2. The effect of decanoate on the P : O ratio obtained for the fumarate-stimulated oxidation of $6.7 \times 10^{-3}$ *M* pyruvate by rat-brain mitochondria. The results obtained in 14 separate experiments are included in this figure.

*Experiments with Rat-brain Mitochondria*

The foregoing results obtained with rat-kidney mitochondria provided conclusive evidence that decanoate, at concentrations of the order of $10^{-3}$ *M*, may uncouple oxidation from phosphorylation. Previous results on the effect of decanoate on the oxidation of pyruvate by rat-brain mitochondria had indicated that pyruvate oxidation per se was unaffected by decanoate whereas the fumarate-stimulated oxidation of pyruvate was sensitive to decanoate. It was of interest, therefore, to compare the effects of decanoate on the oxidative phosphorylations associated with both of these oxidative events. A typical protocol is given in Table II. The decanoate-insensitive oxidation of pyruvate gives rise to little, if any, formation of adenosine triphosphate (ATP) as measured by the uptake of inorganic phosphate. This confirms the observation noted by Brodie and Bain (Table I of Ref. 2). On the other hand, in the presence of both fumarate and pyruvate there is an uptake of inorganic phosphorus, indicating ATP synthesis, and this uptake is decreased in the presence of decanoate. The fumarate-stimulated respiratory activity cannot be decreased, on addition of decanoate, below the level corresponding to the

TABLE II

THE EFFECT OF DECANOATE ON THE PHOSPHATE UPTAKE ASSOCIATED WITH THE OXIDATION OF $6.7 \times 10^{-3}$ *M* PYRUVATE BY RAT-BRAIN MITOCHONDRIA IN THE PRESENCE AND ABSENCE OF FUMARATE

| $2.67 \times 10^{-4}$ *M* decanoate | − | + | − | + |
|---|---|---|---|---|
| $6.7 \times 10^{-4}$ *M* fumarate | − | − | + | + |
| Microatoms $O_2$ taken up | 5.11 | 4.95 | 10.42 | 10.0 |
| Micromoles phosphate taken up | 2.73 | −0.28 | 16.94 | 8.86 |
| $\Delta P/O$ | − | − | 1.63 | 0.89 |
| Corrected $\Delta P$ | − | − | 14.21 | 8.86 |
| $\Delta O$ | − | − | 5.31 | 5.05 |
| Corrected $\Delta P/\Delta O$ | − | − | 2.66 | 1.75 |

rate of oxidation of pyruvate alone. It seems reasonable, therefore, in calculating P : O ratios, to make due allowance for an oxygen consumption, due to the unstimulated pyruvate oxidation, which is not coupled with ATP production. When this is done the P : O ratio increases from approximately 1.5 to approximately 2.5 and is then in harmony with the results obtained with kidney mitochondria. P : O ratios of 2.5–3.0 have been reported for brain mitochondria with pyruvate and malate as substrate but, in general, brain cortex was used in these studies (1, 4). In the present work, mitochondria were prepared from whole cerebral hemispheres and it is possible that the mitochondria from areas other than the cortex may give rise to the "pyruvate blank".

The effect of decanoate concentration on the extent of uncoupling was also nvestigated, using brain mitochondria. Again a linear relationship was observed between the decrease in P : O ratio and the amount of decanoate added (Fig. 2).

## Discussion

The present results confirm the suggestion made by Lehninger (9) that fatty acids may act as uncouplers of oxidation from phosphorylation. Further confirmation of this fact is supplied by the results of Dianzani (3) and Dianzani and Scuro (4), who showed that the production of fatty livers in rats also led to the uncoupling of oxidation from phosphorylation. The mechanism by which decanoate uncouples is more difficult to assess since the actual mechanisms involved in oxidative phosphorylation have not been elucidated nor has the action of such well-known uncoupling agents as 2,4-dinitrophenol (DNOP) and antimycin A been fully explained. However, it has been established that oxidation reactions coupled with ATP synthesis may be affected by more than one method. For example, Hollunger (6) has shown that the inhibition by guanidine of citrate oxidation by rat-liver mitochondria or of glutamate oxidation by rabbit-kidney cortex mitochondria may be reversed on addition of DNOP. The explanation offered is that some

stage in the chain of oxidative phosphorylation reactions is inhibited by guanidine and that DNOP acts at a stage prior to that inhibited by guanidine. A similar conclusion has been reached by Lehninger (10), who states ". . . . . there may be fundamental differences in the mechanisms by which various agents uncouple oxidative phosphorylation in mitochondria". Further, the mechanism by which DNOP stimulates respiration is not known. It seems to be established, however, that DNOP may stimulate the enzyme ATP-ase and that the level of ATP-ase activity may in turn control the mitochondrial respiratory activity (8, 11, 12). The present results indicate that the respiration of rat-brain mitochondria in the presence of pyruvate as substrate does not give rise to a phosphate uptake, i.e., that oxidation of pyruvate to acetate does not lead to ATP synthesis. The stimulation of this respiratory activity by DNOP, reported in the previous paper (13), would thus be difficult to explain in terms of an effect on ATP-ase. However, in all experiments on oxidative phosphorylation, 0.01 *M* fluoride was added to inhibit ATP-ase and in the presence of this amount of fluoride no stimulation of mitochondrial respiratory activity was observed on addition of DNOP. In the absence of fluoride there is probably sufficient dicarboxylic acid formation to allow some oxidation of pyruvate via the tricarboxylic acid cycle, even in the absence of added fumarate. This would lead to ATP synthesis thus permitting a stimulation of ATP-ase activity and hence of respiratory activity.

The effects of decanoate on the oxidation of pyruvate by rat-brain mitochondria are more easily understood than are those of DNOP. The oxidation of pyruvate alone, in which acetate is probably produced, leads to no net ATP synthesis and is not influenced by the addition of decanoate in the presence or absence of fluoride. On the other hand, the increased respiratory activity obtained on addition of small quantities of fumarate to the pyruvate-oxidizing system does lead to ATP synthesis, probably through the functioning of the tricarboxylic acid cycle. This synthesis of ATP is sensitive to the presence of small amounts of decanoate, as reflected by the lowering of the P : O ratio. The presence of higher concentrations of decanoate leads to a value of zero for the P : O ratio and at this level there is a slight effect on the increased respiratory activity. Further increase in the amount of decanoate added leads to an elimination of the increased respiratory activity due to added fumarate.

It must be concluded, therefore, that decanoate and probably other fatty acids are able to act as uncoupling agents, but that they act at a stage in the phosphorylation sequence which is not the same as that influenced by DNOP. The direct proportionality between the extent of uncoupling and the amount of decanoate added seems to indicate the involvement of a physical effect such as a disturbance of the phospholipids involved in oxidative phosphorylation.

## Acknowledgment

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# KINETICS OF THE ENZYMICALLY-CATALYZED OXIDATION OF INDOLEACETIC ACID[1]

By G. A. Maclachlan and E. R. Waygood


## Abstract

A study of the kinetics of the enzymically-catalyzed decarboxylation and oxidation of indoleacetic acid has provided evidence that it is a chain autoxidation initiated and propagated by two enzyme-controlled peroxidations. The following reaction sequence occurs:

| | | | |
|---|---|---|---|
| *Initiation* | $Mn^{2+}$ | $\xrightarrow[+\ ROH]{\text{peroxidase or catalase}}$ | $Mn^{3+}$ |
| *Propagation* | $Mn^{3+} + S{-}COOH$ | $\longrightarrow$ | $Mn^{2+} + H^+ + S{-}COO\cdot$ |
| | $S{-}COO\cdot$ | $\longrightarrow$ | $S\cdot + CO_2$ |
| | $S\cdot + O_2$ | $\longrightarrow$ | $SO_2\cdot$ |
| | $SO_2\cdot + ROH$ | $\xrightarrow[\text{or catalase}]{\text{peroxidase}}$ | $RO\cdot + SO_2H$ |
| | $RO\cdot + H^+ + Mn^{2+}$ | $\longrightarrow$ | $ROH + Mn^{3+}$ |
| *Termination* | $SO_2\cdot +$ enzyme | $\longrightarrow$ | $(SO_2{-}\text{enzyme})$ |
| | $Mn^{3+} + ROH$ | $\longrightarrow$ | $RO\cdot + H^+ + Mn^{2+}$ |
| | $2\ RO\cdot$ | $\longrightarrow$ | products |

where S—COOH = indoleacetic acid; S· = skatole radical; $SO_2\cdot$ = oxidized skatole radical or indole peroxide; $SO_2H$ = final reaction product; ROH = phenolic cofactor, i.e., resorcinol; RO· = semiquinol or aryl radical.


## Introduction

Reaction systems have been described that catalyze the decarboxylation and oxidation of the plant growth hormone indoleacetic acid (IAA) by enzymic (13, 14, 15) and non-enzymic means (11). The reactions of the systems were identical in the following respects: (*a*) Manganic ions were required. (*b*) IAA was completely decarboxylated. (*c*) One equivalent of oxygen was consumed per mole of IAA following decarboxylation. (*d*) The yellow end product from systems at pH 6.0 possessed the same chromatographic $R_f$ value and crystallized from ethanol with the same crystal structure. In the present paper the kinetics of the enzymic system are examined with the supposition that IAA is degraded via the same pathway in both enzymic and non-enzymic systems. The results are therefore interpreted in terms of the free-radical mechanism proposed for the non-enzymic system (11, 12).

To recapitulate the reactions of the non-enzymic system briefly: IAA decomposed in the presence of a manganiversene complex (manganiethylenediaminetetracetate) at pH 6.0 with the reduction of manganic ions

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*Contribution from the Department of Botany, University of Manitoba, Winnipeg, Manitoba.*

and with the release and consumption of one equivalent each of carbon dioxide and oxygen at high oxygen tension (cf. 2). The reaction sequence is summarized in the following equations where IAA is represented as S—COOH:

$$S\text{—}COOH + Mn^{3+} \longrightarrow S\text{—}COO\cdot + H^+ + Mn^{2+}$$

$$S\text{—}COO\cdot \longrightarrow S\cdot + CO_2$$

$$S\cdot + O_2 \longrightarrow SO_2\cdot$$

There are three constituents of the non-enzymic system from which the oxidized skatole radical or indole peroxide ($SO_2\cdot$) could withdraw a hydrogen atom to result in a stable final product ($SO_2H$). IAA rapidly decolorizes at least 15 times its concentration of manganiversene while consuming oxygen; thus the oxidized skatole radical may react with versene to initiate chain reactions in which manganiversene is decomposed. Reaction with water would produce hydroxyl radicals and thereby hydrogen peroxide, but no evidence has been found to substantiate the presence of the latter in the system. In recent experiments (10) it was found that more IAA was always destroyed than theoretically expected when it was incubated with less than equimolar amounts of manganiversene. Manganic ions evidently acted catalytically and the system to some extent propagated itself as an autoxidation. The oxidized skatole radical therefore possesses a high enough redox potential to react with IAA directly, or perhaps indirectly through manganese, i.e.,

$$SO_2\cdot + S\text{—}COOH \longrightarrow SO_2H + S\cdot + CO_2$$

The structure of the final yellow product and proof of its empirical formula, i.e., $SO_2H = C_9H_9O_2N$, have yet to be determined. It must be noted that under certain conditions little or none of the yellow product was formed non-enzymically. Thus anaerobically, and to some extent in air, and even in an oxygen atmosphere below pH 5.0, a purple-black precipitate was formed, which indicated that polymerization had occurred.

Reactions of the oxidized skatole radical are important in interpreting the results of the enzymic system since previous studies (15) indicated that a product of IAA breakdown was utilized to propagate further IAA breakdown, i.e., the enzymic system was cyclic. Complete decarboxylation and oxidation of IAA required well-dialyzed peroxidase or beef liver catalase, manganese, and a specific organic cofactor, e.g., dichlorophenol (DCP), resorcinol, maleic hydrazide, etc. It was established (15) that substances which acted as cofactors in the oxidation of IAA were those which, in an oxidized form, were capable of oxidizing manganese in similar peroxide–phenol–peroxidase systems (9). Therefore we have suggested that a product of IAA breakdown reacts peroxidatically with the organic cofactor, which in turn oxidizes manganese, and manganic ions react with IAA as described above and thus propagate an autoxidation sequence.

In order to test this hypothesis, the effects on the rate of IAA oxidation of varying the concentrations of enzyme, substrate, organic cofactors, and

manganese as well as pH and temperature were examined. Experimental results are also included which provide evidence for the mechanism of initiation reactions.

## Methods

Enzyme extracts were prepared from the leaves of wheat, *Triticum compactum* (Host.) var. Little Club, by direct grinding, centrifugation, and dialysis as previously described (15). Unless otherwise indicated the extracts were dialyzed against 20 vol. of fresh distilled water for each of three consecutive 24 hr. periods.

A natural factor, which was effective in replacing artificial organic cofactors required for IAA oxidation (15), was prepared from the dialyzing medium of the wheat leaf extracts. The medium from the first 24 hr. dialysis was concentrated *in vacuo* at 45° C., acidified to pH 2.0 with phosphoric acid, and extracted with three volumes of ether. After evaporation of the ether, the yellow residue from the ether layer was taken up in water, adjusted to pH 6.0 with NaOH, and made to 1/10 of the volume of the original enzyme extract. Usually 0.15 ml. of this preparation was used in conjunction with 0.5 ml. of enzyme extract in systems for IAA oxidation thus effecting approximately a threefold concentration and purification of the active principle.

Decarboxylation and oxidation of IAA was followed by measuring carbon dioxide release and oxygen uptake in standard respirometers held in a bath at 29.5° C. Reactions were started by the addition of IAA from a side arm to the other system components in the main compartment. When referred to as such the "standard" system for IAA oxidation contained the following constituents at the concentrations indicated in a total volume of 3.0 ml.: enzyme, 0.5 ml. (0.20 mgm. protein *N*); orthophosphate pH 6.0, 150 $\mu$M. ($5 \times 10^{-2}$ *M*); manganous chloride, 3 $\mu$M. ($10^{-3}$ *M*); resorcinol, 1.5 $\mu$M. ($5 \times 10^{-4}$ *M*); IAA, 6.67 $\mu$M. ($2.22 \times 10^{-3}$ *M*).

## Experimental Results

### *1. Enzyme Concentration*

It was shown (15) that with low concentrations of enzyme the progress curves for IAA oxidation attained an equilibrium lower than the theoretical oxygen equivalence. At these low equilibria, peroxidatic activity of the systems could not be detected by the benzidine test and rapid oxidation resumed only if more enzyme were added. Evidently the peroxidatic activity was destroyed or strongly inhibited during IAA oxidation. Similar effects were described by Kenten (8) with the horse-radish peroxidase-catalyzed oxidation of IAA.

In the presence of optimum (see later sections) concentrations of resorcinol and manganese, the effects of enzyme inactivation are shown by systems containing up to 0.25 ml. of enzyme (Fig. 1, C–E) in which theoretical oxygen equivalence was not attained. The change in slope at about 30 min. indicates

the point where the enzyme concentration became limiting. Theoretical amounts of oxygen were consumed when 0.5 ml. of enzyme was used (Fig. 1, B), but higher concentrations of enzyme (Fig. 1, A) decreased the amount of oxygen uptake in this particular experiment. Analogous results were obtained using DCP in place of resorcinol at the same concentration ($5 \times 10^{-4}$ *M*).

The effect of varying the enzyme concentration in the presence of *suboptimum* amounts of resorcinol ($5 \times 10^{-5}$ *M*) are shown for comparison in Fig. 2. As expected, the initial velocities of IAA oxidation were lower at each enzyme concentration than in the corresponding systems containing optimum amounts of resorcinol (Fig. 1). But in addition, lower final equilibria were attained. Thus, for example, with 0.25 ml. of enzyme about 120 $\mu$l. oxygen was consumed at equilibrium (Fig. 1, C) as compared to about 70 $\mu$l. (Fig. 2, C) at the lower cofactor concentration. In similar experiments using the natural factor (concentration unknown, but suboptimum), 0.25 ml. of enzyme catalyzed the consumption of only 40 $\mu$l. oxygen at equilibrium. With the natural factor and limiting resorcinol it was again found that the enzyme was the only component of the systems which, when added at equilibrium, brought about a significant increase in the rate of IAA oxidation. Therefore from a comparison of Figs. 1 and 2 it is evident that more enzyme was inactivated at low cofactor concentrations than at the optimum.

The intermediate causing inactivation appeared to be an oxidized substance since in any one experiment the oxygen consumed at equilibrium was related

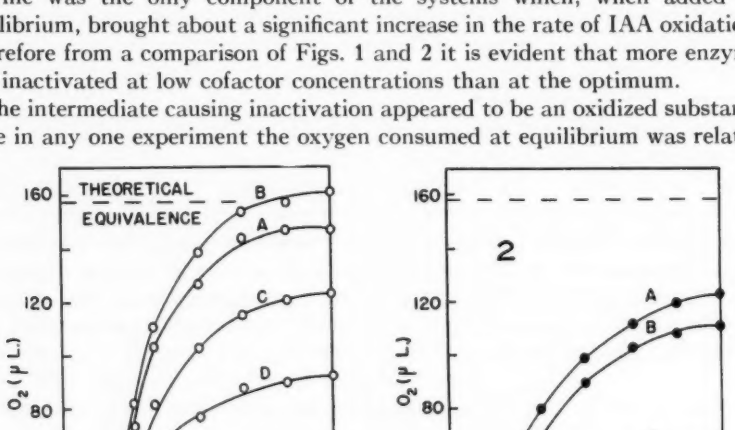


FIG. 1. Effect of enzyme concentration on indoleacetic acid oxidation with optimum cofactor. Enzyme conc. in ml.: A, 1.0; B, 0.50; C, 0.25; D, 0.10; E, 0.05. System (standard): 0.5 ml. enzyme (0.20 mgm. protein N); $5 \times 10^{-4}$ *M* resorcinol; $10^{-3}$ *M* $MnCl_2$; $5 \times 10^{-2}$ *M* phosphate, pH 6.0; $2.22 \times 10^{-3}$ *M* IAA = 158 $\mu$l. $O_2$; final vol. 3.0 ml.; temp. 29.5° C.

FIG. 2. Effect of enzyme concentration with suboptimum cofactor. Systems same as FIG. 1 except resorcinol = $5 \times 10^{-5}$ *M*.

to the amount of enzyme inactivated, e.g., Fig. 1, C–E. But since more enzyme was inactivated at low concentrations of cofactor it must be concluded that optimum amounts of cofactor had a 'protective' effect on the enzyme. Dependence of enzyme "inactivation" on the enzyme/cofactor concentration ratio is satisfactorily explained if it is assumed that the substance which causes inactivation may react *either* with the enzyme *or* with the cofactor. It is suggested that the oxidized skatole radical is the most probable inactivating agent and that in the absence of sufficient hydrogen donor (cofactor) a relatively stable and inactive indole peroxide–enzyme complex is formed.

The enzyme concentration/activity curves for resorcinol, DCP, and the natural factor (Fig. 3) all deviate from linearity at high enzyme concentrations. Similar results were reported in other IAA "oxidase" systems (4, 5, 6). In all of these systems this effect is understandable if it is assumed that the enzyme-inactivating intermediate was also an oxidant of a natural or artificial cofactor and as such promoted the oxidation of manganese and IAA. By increasing the concentration ratio of enzyme/cofactor, more of the intermediate would be diverted from chain oxidation of IAA to inactivation of the enzyme. Thus, because of competition, a linear increase in activity with enzyme concentration would not be expected except at low enzyme/cofactor concentration ratios, i.e., where the enzyme was saturated with hydrogen donor.

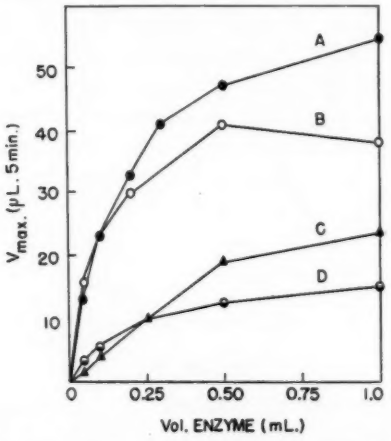


FIG. 3. Effect of enzyme concentration on the oxidation rate with various cofactors. Systems standard with: A, $5 \times 10^{-4}$ *M* dichlorophenol; B, $5 \times 10^{-4}$ *M* resorcinol; C, 0.15 ml. natural factor; D, $5 \times 10^{-5}$ *M* resorcinol. [On the ordinate of this figure, (μl. 5 min.) should read μl./5 min.).]

*2. Substrate Concentration*

A typical set of progress curves for the oxidation of variable amounts of IAA (0.56 to $3.33 \times 10^{-3}$ *M*) is shown in Fig. 4. There are three distinctive features of the curves. Firstly, the lag phase which preceded IAA oxidation was due to incomplete removal of endogenous inhibitors since the enzyme used in this particular experiment had been dialyzed for only eight hours.

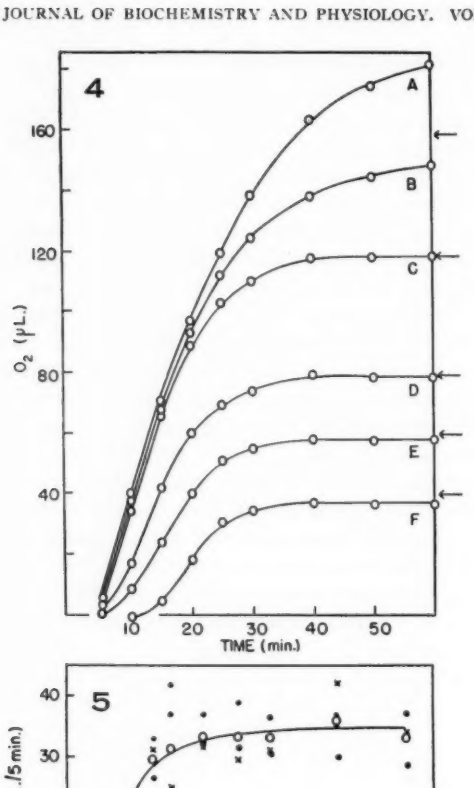


FIG. 4. Effect of indoleacetic acid at various concentrations. Systems standard with IAA in μgm.: A, 10.0; B, 6.67; D, 5.0; D, 3.33; E, 2.5; F, 1.67. Theoretical molar oxygen equivalences for curves B to F indicated by arrows.

FIG. 5. Effect of indoleacetic acid concentration on the oxidation rate. Systems standard, allowed to react in atmospheres of air (solid circles) and oxygen (crosses). Average rates in air and oxygen shown as open circles.

The lag phase was longest in reaction mixtures containing the least amount of IAA thus implicating the substrate as a factor in overcoming inhibitors of its own oxidation. Secondly, the rates of oxygen uptake increased linearly with increasing concentrations of IAA up to about $1.7 \times 10^{-3}$ $M$ (cf. Fig. 5). Thirdly, theoretical oxygen uptake was only attained at equilibrium when $1.7 \times 10^{-3}$ $M$ IAA or less was used. Above this concentration the effects of

enzyme inactivation were evident in the premature decline of reaction velocity (Fig. 4, A, B). Kenten (8) has found similarly that horse-radish peroxidase preparations oxidized small amounts of IAA ($0.83 \times 10^{-3}$ *M*) almost completely, but larger amounts ($3.33 \times 10^{-3}$ *M*) required further additions of enzyme before a comparable destruction of IAA took place.

In order to compare the initial velocities of reactions with lag phases of variable length, the maximum increments attained in any five minute period were chosen as the most reproducible feature of the progress curves. In most cases this was the increment from 5 to 10 min. after oxygen uptake had begun (Fig. 4). The IAA concentration/activity curves obtained in this manner (Fig. 5) were approximately hyperbolic, but variations within any single experiment precluded an accurate kinetic treatment of the data. When the experiment was performed in an oxygen atmosphere, the lag phase was almost overcome, similar initial velocities were obtained, but variability was still encountered. From the average of the maximum velocities found in the experiment in oxygen and three experiments in air (Fig. 5) a Michaelis constant of between 0.7 and $1.1 \times 10^{-3}$ *M* was calculated.

*3. The Effect of pH*

Hydrogen ions are not involved in the over-all reaction of normal peroxidation (3), i.e.:

$$ROOH + AH_2 \longrightarrow ROH + H_2O + A$$

Hence the reaction velocity is constant over a wide pH range. This was not true of IAA oxidation. In the presence of resorcinol, DCP, maleic hydrazide, or the natural factor the rate of oxygen uptake was greatest about pH 5.0 and was markedly inhibited above pH 6.0 (Fig. 6). At pH 4.0 and 6.0,

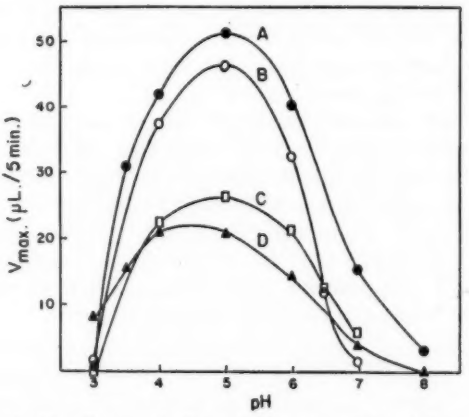


FIG. 6. Effect of pH. Enzyme-buffer mixtures prepared as follows: 1.25 ml. wheat leaf enzyme plus 3.0 ml. 0.1 *M* phosphate adjusted to required pH and made to vol. 5.0 ml. Reaction systems contained: 2.0 ml. enzyme-buffer; $10^{-3}$ *M* $MnCl_2$; cofactor; $2.22 \times 10^{-3}$ *M* IAA; final vol. 3.0 ml. Cofactors as follows: A, $5 \times 10^{-4}$ *M* dichlorophenol; B, $5 \times 10^{-4}$ *M* resorcinol; C, $10^{-2}$ *M* maleic hydrazide; D, 0.15 ml. natural factor.

where the rates of oxidation were suboptimum, the reactions nevertheless proceeded to molar equivalence without showing signs of enzyme inactivation. The rate of carbon dioxide release, measured in one experiment using resorcinol, was somewhat greater than the rate of oxygen uptake at pH 3.0, 4.0, and 5.0, but equal to it at higher pH values. The reaction product at each pH and with each cofactor was a soluble yellow substance. Precipitate formation, so marked in the non-enzymic system below pH 5.0 (11), was never observed.

Inactivity of the system above neutrality can not be due to enzyme inactivation, but more probably to the instability of an essential intermediate, e.g., manganic ions or free radicals. It is also possible that insoluble manganese carbonate is formed at higher pH values (7) thus lowering the effective manganese concentration below optimum. In addition, the oxidation of manganese by cofactor radicals (RO·) involves the utilization of hydrogen ions, i.e.:

$$Mn^{2+} + H^{+} + RO\cdot \rightleftharpoons Mn^{3+} + ROH$$

Since the reverse reaction can readily be demonstrated (15) a high pH would be expected to retard manganese oxidation and hence IAA destruction by a mass action effect.

*4. Temperature*

The rate of IAA oxidation was measured at four temperatures: 10°, 20°, 29.5°, and 42.5° C., using either DCP, maleic hydrazide, the natural factor, or resorcinol as cofactor, the latter in both air and oxygen atmospheres. The initial velocities were represented by the maximum increment of any five minute period rather than the net increment from zero time since with each cofactor the lag phase of at least 10 min., which occurred at the lowest temperature, was almost overcome at the highest. Duplicate experiments were performed and the results, which agreed closely, were averaged to give the temperature/activity curves shown in Fig. 7.

The rate of IAA oxidation increased with increasing temperature in the range 10°–29.5°, but only the systems containing DCP and maleic hydrazide (Fig. 7, A, B) were significantly stimulated by a further temperature increase. At 42.5° C. the progress curves of systems containing the natural factor or resorcinol in air were almost coincident with the corresponding curves at 29.5° C. as indicated in Fig. 7, C, E. When the oxygen tension was increased in the presence of resorcinol the rate of IAA oxidation (Fig. 7, C, D) was increased at 10° C., but was decreased at 42.5° C.

Thermal inactivation evidently began at temperatures above 30° C., particularly in the presence of resorcinol. However, according to the benzidine test, inactivation was not due to enzyme destruction. Instead, IAA autoxidation appeared to be interrupted at higher temperatures by side reactions e.g., cofactor polymerization. The brown color of oxidized resorcinol formed most intensely at high temperatures and in an oxygen atmosphere, prior to the addition of IAA. Hence by promoting condensation of phenolic intermediates,

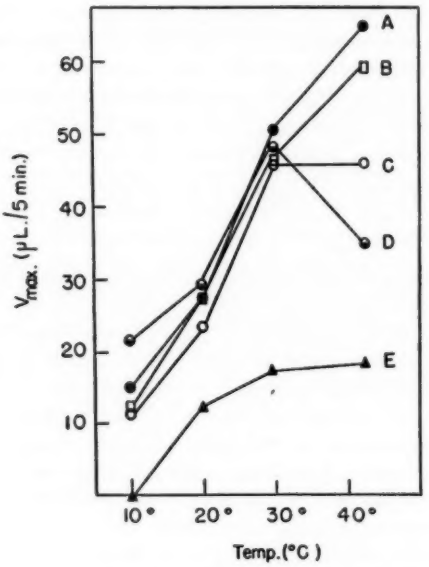


FIG. 7. Effect of temperature. Systems standard with the following cofactors: A, $5 \times 10^{-4}$ *M* dichlorophenol; B, $10^{-2}$ *M* maleic hydrazide; C, $5 \times 10^{-4}$ *M* resorcinol in air; D, $5 \times 10^{-4}$ *M* resorcinol in oxygen; E, 0.15 ml. natural factor.

oxygen and high temperatures diverted an essential intermediate from IAA oxidation, i.e., promoted termination reactions.

From the ratio of the velocities at different temperatures the energy of activation (Table I) was estimated by employing the Arrhenius equation:

$$E_a = T_1T_2/0.219\ (T_2-T_1)\ \log\ V_2/V_1$$

Since IAA oxidation is a chain reaction, it must be taken into consideration that temperature probably influences initiation and termination as well as propagation reactions. The fact that the lag phase was variable indicated that initiation reactions were temperature controlled. Indeed, at 10° C. IAA was stable for a period of at least one hour in the presence of the natural factor (Fig. 7, E), which, as used in these experiments, contained inhibitors of initiation as well as activators of propagation (15). Accordingly, the activation energy for the natural-factor-catalyzed system would be infinitely large if calculated from the interval 10°–20° C. On the other hand, with termination reactions prominent at higher temperatures, $E_a$ values calculated from the interval 29.5°–42.5° C. were low and even negative with resorcinol in oxygen.

The activation energy of the system as a chain sequence, i.e., propagation reactions, should be most closely approximated by comparing the velocities at intermediate temperatures. From 20°–29.5° C. the values for $E_a$ in the

TABLE I

ARRHENIUS ACTIVATION ENERGY OF IAA OXIDATION WITH VARIOUS COFACTORS

| Temperature interval | Activation energy (cal./mole) in the presence of: | | | | |
|---|---|---|---|---|---|
| | DCP | Maleic hydrazide | Resorcinol in air | Resorcinol in oxygen | Natural factor |
| 10°–20° C. | 10,600 | 12,800 | 11,800 | 5,300 | — |
| 20°–29.5° C. | 7,700 | 7,100 | 8,200 | 6,800 | 6,000 |

presence of all cofactors were close and averaged 7160 cal./mole (Table I). This corresponds to a $Q_{10}$ from 20°–30° C. of about 1.5. It is interesting that the lowest $E_a$ value (6000 cal./mole) in this temperature interval was obtained from the system containing the natural factor, which on this basis may be considered the most efficient mediator. From 10°–20° C. the $E_a$ values were higher (averaging *ca.* 11,700 cal./mole) in the presence of DCP, maleic hydrazide, and resorcinol in air but lower (5300 cal./mole) with resorcinol in oxygen. The high values probably reflect difficulty in overcoming natural inhibitors of the reaction that are destroyed by increasing oxygen tension (15) thus resulting in a low $E_a$ value.

*5. Cofactor Concentration*

The oxidation of IAA catalyzed by dialyzed wheat leaf extracts and manganese was stimulated by the addition of certain organic cofactors, e.g., resorcinol and monohydric phenols; however, there was invariably some endogenous activity in the absence of a cofactor (Fig. 8). This was also true

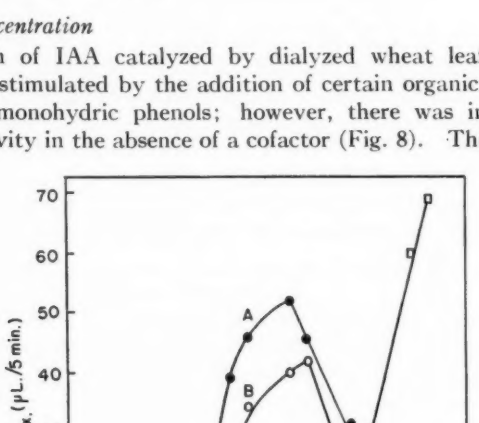


FIG. 8. Effect of cofactor concentration on the oxidation rate. Systems standard with the following cofactors: A, dichlorophenol; B, resorcinol; C, maleic hydrazide.

of ammonium sulphate precipitates of wheat leaf extracts as well as horseradish peroxidase preparations. The endogenous activity could be decreased by extended dialysis, but preparations which were *completely* inactive in the absence of an added cofactor were never obtained even after the protein was washed with acetone followed by dialysis. It was concluded that peroxidase has a marked affinity for naturally-occurring substances which act as redox catalysts in the oxidation of manganese. Incomplete removal of such substances probably accounts for the IAA "oxidase" activity of extracts from various plant sources to which no cofactor was added.

The cofactor concentration/activity curves for resorcinol and DCP (Fig. 8, A, B) showed optima at $10^{-3}$ *M* and $5 \times 10^{-4}$ *M* respectively in the presence of $10^{-3}$ *M* manganese. Above these concentrations the activity of the systems fell rapidly to zero. In contrast, higher concentrations of maleic hydrazide, i.e., from $10^{-3}$ *M* to its maximum solubility (*ca.* $10^{-1}$ *M*), were not inhibitory, but continued to promote IAA oxidation (Fig. 8, C).

It has been argued (15) that for a substance to act as a cofactor it must be a substrate for peroxidase or catalase (reaction A, *vide infra*) and act as a redox catalyst for the oxidation of manganese (reaction B). The intermediate which enters into a peroxidatic oxidation with the cofactor (ROH) is considered to be the oxidized skatole radical ($SO_2\cdot$) and the resulting aryl radical intermediate ($RO\cdot$) is then capable of oxidizing manganese.

$$\text{(A)}\quad SO_2\cdot + ROH \xrightarrow[\text{or catalase}]{\text{peroxidase}} SO_2H + RO\cdot$$

$$\text{(B)}\quad RO\cdot + H^+ + Mn^{2+} \rightleftharpoons ROH + Mn^{3+}$$

The reversibility of reaction B has been demonstrated by incubating resorcinol and DCP (ROH) with manganic ions in complex with versene and observing the rate of decolorization of manganiversene (15). Resorcinol forms a brown and DCP a white oxidation product both of which presumably result from condensation reactions, i.e.: $2RO\cdot \longrightarrow$ products.

The abrupt decline shown in the concentration/activity curves for DCP and resorcinol systems (Fig. 8, A, B) resembles the fall off in activity of most enzymic systems at the extremes of pH or temperature; the fall off, in these cases, is due to enzyme inactivation. However, at high phenol concentrations there was little or no evidence of enzyme inactivation although the progress curves for oxygen uptake attained equilibria lower than the theoretical. At equilibrium, benzidine tests were still positive and no further IAA oxidation occurred if enzyme were added (compare the reverse results at low phenol concentrations, i.e., Fig. 2, p. 1236). Evidently a premature decline in reaction velocity cannot be taken as necessarily diagnostic of enzyme inactivation.

Accordingly, inhibition of IAA oxidation by excess resorcinol and DCP may be explained by a mass action effect in reaction B. An excess of phenolic cofactor would compete with IAA for manganic ions. Thus, the equilibrium

constant of the redox system phenol/manganese controls the rate of the entire autoxidation sequence at supraoptimum levels of phenol, whereas the phenol/enzyme ratio controls at suboptimum levels.

On the other hand if manganic ions were not reduced by the cofactor, i.e., if reaction B were not reversible, then inhibition at high concentrations should not occur. Maleic hydrazide provides an example of this type of cofactor and its reaction with manganiversene has never been detected visibly (15) or spectrophotometrically (10). Accordingly, maleic hydrazide does not compete with IAA for manganic ions and therefore with increasing concentrations continues to accelerate IAA oxidation. It is suggested that if a particular cofactor reacts with manganic ions it will exhibit an optimum concentration in its ability to promote IAA oxidation. From a brief survey of known Kenten–Mann cofactors (9) this would apply to phenol, *o*-cresol, *p*-phenylenediamine, and *p*-chlorophenol, all of which decolorize manganiversene.

The over-all rate of IAA oxidation may be determined either by the equilibrium established in reaction B or by the rate of enzymic peroxidation (reaction A). For example, maleic hydrazide must be difficult to oxidize peroxidatically (reaction A) since considerably higher concentrations (100-fold) are required to produce an activity comparable to that of either resorcinol or DCP. When equimolar with resorcinol, DCP was a more efficient cofactor at all concentrations. Accordingly, either DCP was more efficiently oxidized by peroxidase than resorcinol (reaction A) or it reduced manganic ions more slowly (reaction B), or both factors contributed to the efficiency of DCP. Qualitative experiments with manganiversene showed that DCP reduced manganic ions more slowly than did resorcinol. Hence, in net effect the radical of DCP probably provided manganic ions to IAA at a faster rate than did the semiquinol of resorcinol because of a higher equilibrium constant in reaction B.

On the premise that an intermediate product of IAA oxidizes resorcinol it would be expected that an increase in the concentration of IAA would increase the ratio of oxidized/reduced resorcinol and thereby overcome inhibition by excess of the reduced form, i.e., owing to reversal of reaction B. Thus the oxidation of $2.2 \times 10^{-3}$ *M* IAA was completely inhibited by the presence of $5 \times 10^{-2}$ *M* resorcinol (Fig. 8, B), but when the initial concentration of IAA was increased 12-fold to $2.67 \times 10^{-2}$ *M*, oxygen uptake proceeded for at least one hour at an average rate of 27 $\mu$l./5 min. Therefore, the system was potentially active in the presence of $5 \times 10^{-2}$ *M* resorcinol, but its activity was dependent on the concentration ratio IAA/resorcinol. Accordingly, it might be more accurate to describe the optimum concentrations of resorcinol and DCP as relative to the IAA concentration, i.e.: IAA/resorcinol = 2.2, IAA/DCP = 4.4.

*6. Manganese Concentration*

The effect of varying the concentration of manganese from $10^{-6}$ *M* to $10^{-1}$ *M* on the rate of oxidation of IAA is shown in Fig. 9. Succinate buffer

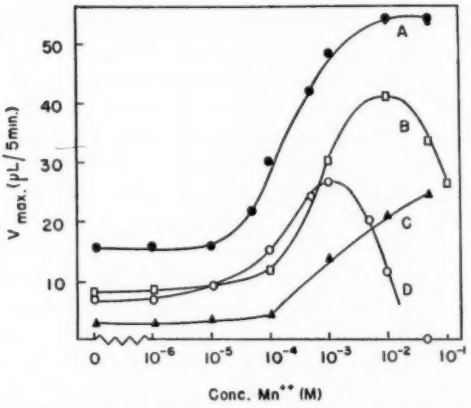


FIG. 9. Effect of manganese concentration of the oxidation rate. Systems standard with $5 \times 10^{-2}$ *M* succinate buffer pH 6.0 and the following cofactors: A, $5 \times 10^{-4}$ *M* dichlorophenol; B, $10^{-2}$ *M* maleic hydrazide C, 0.15 ml. natural factor; D, $5 \times 10^{-4}$ *M* resorcinol.

was used throughout in order to avoid precipitation of insoluble manganese salts and consequent alteration in pH. The velocities at concentrations of manganese above $10^{-4}$ *M* are averages of at least two experiments.

In the absence of added manganese, oxidation of IAA proceeded slowly in spite of extensive dialysis of the enzyme preparation against water to remove endogenous manganese. The actual rate in the absence of manganese depended on the cofactor used. Of those tested DCP was the most effective yielding a maximum initial velocity of 16 μl. $O_2$/5 min. (Fig. 9, A). After further dialysis of the enzyme against $2.5 \times 10^{-4}$ *M* versene for 24 hr. the rate was decreased to 3.5 μl. $O_2$/5 min. Further dialysis against water instead of dilute versene did not diminish the endogenous rate. This may be taken as evidence for the ability of the protein of these preparations to bind manganese and also the special properties of DCP as a mobilizing or coordinating agent of endogenous manganese.

In general, increasing the manganese concentration did not prolong the lag phase, but had either of two effects on the rate of oxygen uptake depending on the cofactor used. In the presence of the natural factor or DCP (Fig. 9, A, C) IAA was oxidized most rapidly at the highest concentration of manganese ($10^{-1}$ *M*) although with DCP no further stimulation occurred beyond $10^{-2}$ *M* manganese. In contrast, with resorcinol and maleic hydrazide, distinct optimum manganese concentrations were found at $10^{-3}$ *M* and $10^{-2}$ *M* respectively (Fig. 9, B, D).

Inhibition by excess manganese does not involve an inactivation of the enzyme nor an interference in the sequence of reaction undergone by IAA since neither phenomenon takes place in the presence of DCP or the natural factors (Fig. 9). Furthermore, increasing the concentration of IAA 12-fold failed to overcome manganese inhibition as it overcame inhibition by excessive

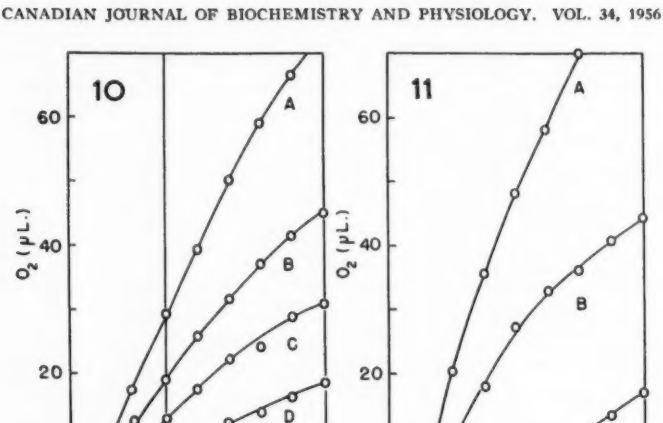


FIG. 10. Effect of resorcinol concentration on its rate of oxidation by manganese–peroxidase systems. Systems contained: 0.50 ml. wheat leaf enzyme; $5 \times 10^{-2}$ *M* $MnCl_2$; $5 \times 10^{-2}$ *M* succinate buffer pH 6.0; final vol. 3.0 ml. Resorcinol molarity as follows: A, $10^{-1}$; B, $5 \times 10^{-2}$; C, $10^{-2}$; D, $5 \times 10^{-3}$; E, $10^{-3}$. $2.22 \times 10^{-3}$ *M* IAA added to all systems at 30 min.

FIG. 11. Effect of manganese concentration on resorcinol oxidation. Systems contained 0.5 ml. wheat leaf enzyme; $5 \times 10^{-2}$ *M* resorcinol; $5 \times 10^{-2}$ *M* succinate buffer pH 6.0; final vol. 3.0 ml. Manganese molarity as follows: A, $5 \times 10^{-2}$; B, $10^{-1}$; C, $10^{-2}$.

resorcinol. Therefore it does not appear that manganous ions are protecting IAA by, for example, displacing manganic ions from complex with IAA. Addition of cofactors also had no effect on manganese-inhibited IAA oxidation (see later, Fig. 11) although this would not be expected from any mass action effect in reaction B (p. 1243).

The fact that the optimum manganese concentrations ($10^{-3}$ *M* and $10^{-2}$ *M*) were close to the concentrations of resorcinol ($5 \times 10^{-4}$ *M*) and maleic hydrazide ($10^{-2}$ *M*) appeared to be coincidental. The optima remained unchanged when experiments were performed using less cofactor, i.e., $5 \times 10^{-5}$ *M* resorcinol and $5 \times 10^{-3}$ *M* maleic hydrazide. Accordingly, excess manganese cannot inhibit by reversing, but rather by interrupting, an essential reaction, e.g., an oxidation, involving the organic cofactors. It is noteworthy that excess manganese also inhibited the oxidation of resorcinol (resorcinol "oxidase") which occurs independently of IAA in the presence of manganese and peroxidase (see later, Fig. 11). It therefore appears justifiable to suggest that this occurs during IAA oxidation, but as yet the mechanism of manganese inhibition is not clear.

### 7. *Initiation Reactions*

The lag or induction period which occurs in all progress curves for IAA decarboxylation and oxidation has been considered to represent the time

necessary for the system to build up a threshold concentration of manganic ions (14). After induction the reaction exhibits autocatalytic characteristics which indicate that the products of IAA breakdown contribute to initiation reactions. However, to account for the initiation of the reaction it is necessary to postulate that oxidizing power arose in the extracts prior to and independently of the process of IAA breakdown. Presumably this oxidizing power was effective in the form of manganic ions generated by reaction of the other system components, i.e., enzyme and cofactor. The independent reactions of resorcinol, enzyme, and manganese were therefore investigated.

The ability of wheat leaf extracts to oxidize resorcinol was first noted in experiments performed in an oxygen atmosphere where, prior to addition of IAA, the reaction mixtures developed the brown color characteristic of irreversibly oxidized resorcinol (15). This was less evident in air, but final reaction mixtures always included the same brown substance which was presumably formed by semiquinol condensation. A similar "oxidase" reaction occurred with purified horse-radish peroxidase or beef liver catalase and no color was formed in the absence of manganese. The oxidation cannot be due to polyphenol oxidase since this enzyme is not present in wheat leaves.

Oxygen consumption in this system was investigated by adding resorcinol at concentrations of between $10^{-1}$ $M$ and $10^{-3}$ $M$ to reaction mixtures containing wheat leaf extracts and $5 \times 10^{-2}$ $M$ manganese in succinate buffer. After a lag phase of 5–10 min. duration, oxygen uptake began at rates proportional to the concentration of resorcinol (Fig. 10). This system will be referred to as the resorcinol "oxidase" system.

From previous kinetic data one would not expect IAA to be oxidized by these resorcinol "oxidase" systems owing to the high level of manganese (cf. Fig. 9, D). That this was the case is shown in Fig. 10 where IAA, added after 30 min., had no effect on the progress of oxygen uptake. After two hours, Salkowski tests confirmed that IAA was not destroyed. Evidently inhibition of IAA oxidation by excess manganese was not overcome by raising the concentration of resorcinol.

The effect of the manganese concentration on the resorcinol "oxidase" system is shown in Fig. 11. Manganese was most effective in promoting oxygen uptake when equimolar ($5 \times 10^{-2}$ $M$) with resorcinol (Fig. 11, A). At higher ($10^{-1}$ $M$) or lower ($10^{-3}$ $M$) concentrations of manganese (Fig. 11, B, C respectively) the system was considerably less active. In separate experiments it was again found that IAA was not oxidized at these cofactor concentrations.

The rate of oxygen consumption by resorcinol was greatly enhanced and the lag phase was decreased by replacing air in the reaction vessels with oxygen. The lag phase in air or oxygen was abolished by adding either riboflavin-phosphate, hydrogen peroxide, or manganiversene. Furthermore citrate and pyrophosphate completely inhibited resorcinol oxidation, all of which suggests that manganic ions were involved. Further studies on the system in an oxygen atmosphere have established that the optimum pH lay between 5.0

and 6.0 and that the oxygen consumed at equilibrium exceeded 1 mole per mole of resorcinol. The reaction appeared to be complex and to form a mixture of products in various states of oxidation.

Catalase or peroxidase, with manganese, were the only apparent requirements of the resorcinol "oxidase" system, but it is interesting to note that oxygen was consumed at a slow rate in the presence of riboflavin or its phosphate and manganese, but in the absence of enzyme. This non-enzymic oxidation, unlike the resorcinol "oxidase" system, was stimulated by light.

Certain aspects of these systems resemble the system described by Andreae (1), which forms manganipyrophosphate from solutions containing catalase, riboflavin, a monohydric phenol or resorcinol, manganous ions, and pyrophosphate. However, Andreae's system required light as well as each cofactor for maximum manganese oxidation. Light had no effect on resorcinol "oxidase" and riboflavin did not appear to be necessary. Nevertheless it is possible that both systems developed oxidizing power by similar mechanisms. Whether oxidized resorcinol or manganic ions were the end product appeared to depend only on the presence of an acceptor for manganic ions. In Andreae's system this was pyrophosphate or citrate. It is suggested that in the resorcinol "oxidase" system little irreversible resorcinol oxidation would occur and manganic ions would be reduced if IAA were added, provided the concentration of resorcinol were not high enough to compete successfully with IAA and also if manganous ions were not in excess. In the standard system for IAA oxidation these conditions are fulfilled. The relative concentrations of resorcinol and manganese (1 : 2) are such that some manganese oxidation would be expected (cf. Figs. 10, 11). Also since IAA is present at a high concentration relative to resorcinol (4.4 : 1) it would tend to react preferentially with manganic ions (cf. Fig. 10). Thus the autocatalytic production of manganic ions via the resorcinol "oxidase" system, even if operating to an almost negligible extent in air, could be responsible for initiating IAA oxidation.

The apparent ease with which the semiquinol of resorcinol forms stable condensation products probably accounts for the ability of this phenol to consume oxygen in the above system. Other cofactors, e.g., phenol, *o*-cresol, DCP, maleic hydrazide, did not result in oxygen uptake under the same conditions. These substances have not been studied further but are known to produce manganic ions without the necessity of oxygen uptake (1).

## Discussion and Conclusions

The enzymic oxidation of IAA has been investigated on the assumption that IAA decarboxylates and consumes oxygen via the same reaction sequence that occurs non-enzymically in the presence of manganiversene. The product and stoichiometry of the two reactions are the same but one significant difference has been found between the reaction conditions necessary to achieve equivalence. As shown in the IAA concentration/activity curve (Fig. 5) and the temperature/activity curve (Fig. 7), increasing oxygen tension did not increase the Michaelis constant of the enzymic system nor the rate and

amount of oxygen uptake at 29.5° C. In contrast, a high oxygen tension was essential in the non-enzymic system for complete oxidation (11) even though complete decarboxylation may have taken place.

In the presence of manganiversene the decarboxylation product (skatole radical: S·) polymerized (reaction 1) more readily than it reacted with oxygen (reaction 2), i.e.:

$$(1)\quad \begin{cases} S\cdot + S\cdot \longrightarrow \text{products} \\ S\cdot + IAA \longrightarrow \text{products} \\ S\cdot + MnV^{-} \longrightarrow \text{products} \end{cases}$$

$$(2)\quad S\cdot + O_2 \longrightarrow SO_2\cdot$$

Polymerization has never been observed in the enzymic system. However, this can not be due to a slow rate of formation of the skatole radical (S·) since the most rapid rate of IAA decarboxylation observed non-enzymically (69 $\mu$l. $O_2$/5 min.) was equalled by the rates of some enzymic systems, e.g., Fig. 8. Therefore, whether the skatole radical polymerizes or reacts with oxygen must depend on its rate of disposal, which in turn will depend on the disposal rate of the oxidized skatole radical. Thus reaction (2) may be considered reversible and to proceed rapidly to the right only if a substance is present which reacts with $SO_2$· more rapidly than S· polymerizes.

Evidently formation of the final yellow product from the oxidized skatole radical is more readily accomplished in the enzymic than in the non-enzymic system. The only constituent of the former which is easily oxidized and not present in the latter is an organic cofactor, e.g., resorcinol. Therefore we suggest that the oxidized skatole radical undergoes a direct and rapid enzyme-catalyzed reaction with the organic cofactor rather than what must be a slower reaction with any substance common to both enzymic and non-enzymic systems. This conclusion renders a reaction between the oxidized skatole radical and water improbable in the enzymic system and may be used as further evidence that hydroxyl radical or hydrogen peroxide are not involved.

These conclusions and those arrived at within the text of the preceding sections are summarized in the following reaction sequence for IAA autoxidation by a "standard" system, i.e.: IAA = S—COOH; skatole radical = S·; indole peroxide = $SO_2$· (oxidized skatole radical); product = $SO_2H$; resorcinol = ROH.

*Initiation*

$$Mn^{2+} \xrightarrow[+\ ROH]{\text{peroxidase or catalase}} Mn^{3+}$$

*Propagation*

$$Mn^{3+} + S\text{—}COOH \longrightarrow Mn^{2+} + H^{+} + S\text{—}COO\cdot$$

$$S\text{—}COO\cdot \longrightarrow S\cdot + CO_2$$

$$S\cdot + O_2 \longrightarrow SO_2\cdot$$

$$SO_2\cdot + ROH \xrightarrow[\text{or catalase}]{\text{peroxidase}} RO\cdot + SO_2H$$

$$RO\cdot + H^{+} + Mn^{2+} \longrightarrow ROH + Mn^{3+}$$

*Termination*

$$SO_2\cdot + \text{enzyme} \longrightarrow (SO_2\text{—enzyme})$$

$$Mn^{3+} + ROH \longrightarrow RO\cdot + H^{+} + Mn^{2+}$$

$$2\ RO\cdot \longrightarrow \text{products}$$

The propagation reactions allow for only one manganic ion produced per molecule IAA destroyed. As previously pointed out (15) the system would have to be 100% efficient if there were no other source of manganic ions or propagation pathway. Excess manganic ions could be generated by the resorcinol "oxidase" system. It is also possible that a direct reaction occurs between the oxidized skatole radical and IAA as has been observed in the non-enzymic system (see Introduction). However, as long as the supply of reduced cofactor is sufficient, this reaction is improbable, i.e.: $SO_2\cdot$ reacts with the cofactor more rapidly than with any other system component (see above). We have concluded that the system *can be* close to 100% efficient and that less than complete oxidation occurs only if termination reactions are promoted by conditions which are not optimum.

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# FURTHER STUDIES ON THYROID – ADRENAL – ASCORBIC ACID RELATIONS IN ANIMALS EXPOSED TO COLD[1]

By André DesMarais[2]

## Abstract

The effect of ascorbate administration on resistance to cold and thyroid function was studied in adrenalectomized rats receiving various amounts of cortisone and/or DCA; in thyroidectomized rats given different doses of of thyroxine, the effect of ascorbate administration was evaluated on both resistance to cold and adrenal function.

In adrenalectomized rats given large doses of cortisone (2.5 mgm.) and exposed to cold, the administration of either ascorbate (150 mgm.) or DCA (2.5 mgm.) enhances the survival, reduces the extent of thymolysis, and decreases the activation of the thyroid. In adrenalectomized rats receiving no cortisone or DCA or low doses (0.4 and 0.1 mgm.) of these hormones, ascorbate administration (150 mgm.) still retains some of its beneficial effects on resistance to cold (better growth and survival); when low doses of DCA (0.1 mgm.) are given without cortisone, ascorbate administration seems to have a deleterious effect on the growth and survival during exposure to cold, with a greater increase in thyroid activity.

In thyroidectomized rats exposed to cold, ascorbate administration (150 mgm.) has no effect in the absence of thyroxine, but increases the efficiency of low doses (3 μgm.) of thyroxine, preventing at the same time some of the typical signs of an alarm reaction: thymolysis and adrenal enlargement.

These results are interpreted as showing that the role of the cortical hormones in resistance to cold might be limited to a "conditioning" action and that the beneficial effects of ascorbate administration would be mediated through the thyroid hormones.

In a recent paper (7), we reviewed the work of Dugal *et al.* on the beneficial effects of ascorbic acid administration in animals exposed to cold, and we confirmed their findings concerning the reduction in thymic involution and the prevention of adrenal hypertrophy in rats exposed to cold and given ascorbic acid (10, 18). In the same paper, we confirmed our earlier results (8) showing that these particular effects of ascorbic acid administration were accompanied by a decrease of thyroid activation. We also presented evidence to show that the administration of thyroxine in adequate amounts to thyroidectomized rats exposed to cold duplicates the beneficial effects of ascorbate in normal rats exposed to cold, namely a better growth and the prevention of both thymolysis and adrenal hypertrophy. When given to thyroidectomized–adrenalectomized rats exposed to cold and receiving large doses of cortisone, thyroxine completely reversed the catabolic effect of cortisone. In the same paper, it was shown that ascorbic acid administration had no effect on the growth rate of thyroidectomized–adrenalectomized rats exposed to cold and given large doses of cortisone but no thyroxine.

The question then arose whether the presence of the thyroid or its hormones was necessary to obtain the desired beneficial effect of ascorbate administration in cold-exposed animals. The present paper will deal with some aspects of this problem.

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## I. Adrenalectomized Animals

### A. High Doses of Cortisone and DCA

*1. Experimental*

Sixty male albino rats of the Wistar strain were used. Their weight at the onset of the experiment ranged from 142 to 158 gm. On day 0 of the experiment, 52 animals were operated on and the treatments were initiated 24 hr. later, according to the distribution shown in Table I. All adrenalectomized rats were given 1% sodium chloride in tap water to drink ad libitum. Cortisone and DCA were administered daily at the dosage level indicated, in one subcutaneous injection, while the sodium ascorbate was given by mouth in two daily doses of 75 mgm. in 0.5 ml. of redistilled water. The animals were kept in a constant temperature room at 24 $\pm$ 1.0° C., except when they were exposed to cold in a room maintained at 14 $\pm$ 1.0° C. The body weights were recorded on the 7th and the 14th days of the experiment. At autopsy, the thymus was weighed to 0.1 mgm. on a torsion balance, the thyroid was fixed in Bouin's, and 5 $\mu$ sections were stained with Mallory's triple stain. The height of the epithelium was measured with an ocular micrometer in 10 follicles chosen at random.

*2. Results (Table I)*

In the cortisone-treated rats kept at room temperature, the growth is about one third of normal, the weight of the thymus is significantly reduced ($p < 0.001$), indicating an excessive dose of cortisone, but the thyroidal epithelium remains unchanged.

When cortisone-treated rats are exposed to cold, the growth rate is not further reduced, but the survival is decreased to 69%, the thymus weight is reduced to a significantly greater extent ($p < 0.001$), and the height of the epithelial cells of the thyroid is increased ($p < 0.001$).

In the cortisone-treated, cold-exposed animals, the administration of either ascorbate or DCA enhances the survival, reduces the extent of thymolysis, and significantly ($p < 0.001$) decreases the height of the thyroidal epithelium, as compared to the cortisone-treated animals exposed to cold, without ascorbate or DCA, though the thymus weight and the epithelium height remain significantly different from normal.

### B. Low Doses of Cortisone and DCA

*1. Experimental*

On day 0 of the experiment, 124 male albino rats of the Wistar strain were adrenalectomized. Their initial weight was between 138 and 164 gm. All animals were killed after 20 days; the thymus was weighed and the thyroid removed, prepared, and examined as described in the first experiment. The different treatments, temperature, and time of exposure are indicated in

TABLE I

EFFECTS OF ASCORBATE ADMINISTRATION IN ADRENALECTOMIZED RATS RECEIVING HIGH DOSES OF CORTICOSTEROIDS AND EXPOSED TO COLD

| Treatment | No. animals at autopsy | Growth in gm. | | % survival | Thymus, fresh wt., mgm. | Thyroid, height of epithelium, μ |
|---|---|---|---|---|---|---|
| | | 0–7th day | 7–14th day | | | |
| Untreated, sham-operated rats | 8 | 35.0 | 31.4 | 100 | 367.8 ± 24.72* | 8.24 ± 0.18* |
| Adrenalectomized | | | | | | |
| Cortisone (2.5 mgm.) | 10 | 12.1 | 17.3 | 100 | 182.7 ± 28.53† | 8.31 ± 0.14 |
| Cortisone (2.5 mgm.) + cold: 14° C. (7–14 d.) | 9 | 11.6 | 18.1 | 69 | 62.3 ± 9.41†, ‡ | 12.72 ± 0.32† |
| Cortisone (2.5 mgm.) + ascorbate (150.0 mgm.) + cold: 14° C. (7–14 d.) | 14 | 11.0 | 14.2 | 78 | 123.6 ± 31.32† | 9.78 ± 0.28§ |
| Cortisone (2.5 mgm.) + DCA (2.5 mgm.) + cold: 14° C. (7–14 d.) | 10 | 14.2 | 14.6 | 91 | 176.8 ± 25.63† | 10.65 ± 0.33†, § |

**Standard error of the mean.*
*†Significantly different from untreated, sham-operated rats.*
*‡Significantly different from cortisone-treated rats at room temperature.*
*§Significantly different from cortisone-treated rats exposed to cold.*

Table II. The sodium chloride was administered at 1% concentration in the drinking water; cortisone and DCA were given in one daily subcutaneous injection at the respective dosage levels of 0.4 and 0.1 mgm.; the sodium ascorbate was administered by mouth in two daily doses of 75 mgm. In all these experiments, the changes in temperature and time of exposure to cold were made so that the animals exposed to cold without ascorbate treatment would have approximately the same relative difference in growth rate as compared to their controls at room temperature.

*2. Results (Table II)*

*NaCl With or Without Ascorbate*

Analysis of results show that ascorbate treatment has no particular effect at room temperature. In animals exposed to cold, the impairment of growth is lessened by ascorbate treatment, the decrease in growth being significant only for those without ascorbate ($p < 0.01$). It can also be seen that ascorbate administration increased the survival and prevented the activation of the thyroid. No definite effect was observed on the thymus weight.

*Cortisone and DCA With or Without Ascorbate*

Here again, there is no effect of ascorbate administration in animals kept at room temperature. It has to be noted that in order to obtain the same relative impairment of growth in these animals, where low doses of both cortisone and DCA are substituted for NaCl, we had to use lower temperatures. Despite this, the survival is much better (100%), although ascorbate treatment did not affect the relative growth more than it did in the previous experiment with only NaCl administration. No effect of ascorbate treatment can be observed on the thyroid; exposure to cold increases the activity of this gland in both groups, whether or not they are given ascorbate. As far as the thymus is concerned, there is a small but non-significant decrease in its weight when no ascorbate is given to rats exposed to cold.

*DCA With or Without Ascorbate*

Substituting DCA for NaCl seems to afford a greater degree of resistance to cold, since the survival is better (83% as compared to 46%) at lower temperatures. Moreover, DCA alone appears to be as effective as DCA and cortisone as far as survival is concerned, though the absolute growth is decreased by omission of cortisone.

The most striking observation is the apparent deleterious effect of ascorbate administration in animals exposed to cold and given DCA without cortisone. Again, it is only in the cold that this effect of ascorbate administration is disclosed; at room temperature, ascorbate treatment slightly increases the growth rate of DCA-treated animals.

Another impressive observation is the great increase in thyroid activity in those animals exposed to cold and given this low dose of DCA with or without ascorbate administration. Not only the DCA-treated rats exposed to cold

TABLE II

EFFECTS OF ASCORBATE ADMINISTRATION IN ADRENALECTOMIZED RATS RECEIVING LOW DOSES OF CORTICOSTEROIDS AND EXPOSED TO COLD

| NaCl, 1% | Cortisone, 0.4 mgm. | DCA, 0.1 mgm. | Ascorbate, 150 mgm. | No. animals at autopsy | Temperature and duration of exposure | Growth: Absolute in gm. | Growth: % of controls at room temp. | % survival | Thymus, fresh wt., mgm. | Thyroid, height of epithelium, μ |
|---|---|---|---|---|---|---|---|---|---|---|
| + | | | | 8 | 24° C. (0–20 days) | 61.2 ± 5.2* | | 100 | 628.7 ± 52.9* | 12.5 ± 0.34* |
| + | | | + | 8 | 24° C. (0–20 days) | 62.0 ± 5.8 | | 100 | 626.8 ± 57.6 | 11.3 ± 0.29 |
| + | | | | 6 | 14° C. (0–16 d.); 9° C. (16–20 d.) | 35.0 ± 6.5 | 57.2† | 46 | 550.5 ± 51.8 | 14.4 ± 0.32† |
| + | | | + | 10 | 14° C. (0–16 d.); 9° C. (16–20 d.) | 46.6 ± 8.7 | 75.0 | 83 | 520.8 ± 53.7 | 12.1 ± 0.32 |
| | + | + | | 8 | 24° C. (0–20 d.) | 91.1 ± 6.6 | | 100 | 602.1 ± 48.3 | 12.6 ± 0.27 |
| | + | + | + | 8 | 24° C. (0–20 d.) | 84.1 ± 6.8 | | 100 | 654.9 ± 50.9 | 12.3 ± 0.30 |
| | + | + | | 10 | 10° C. (0–8 d.); 1.5° C. (8–20 d.) | 46.8 ± 6.7 | 51.4† | 71 | 494.4 ± 46.6 | 13.8 ± 0.32† |
| | + | + | + | 13 | 10° C. (0–8 d.); 1.5° C. (8–20 d.) | 60.8 ± 7.3 | 72.4 | 100 | 670.3 ± 54.5 | 14.3 ± 0.34† |
| | | + | | 8 | 22° C. (0–20 d.) | 60.0 ± 5.5 | | 100 | 618.2 ± 51.4 | 12.9 ± 0.28 |
| | | + | + | 8 | 22° C. (0–20 d.) | 72.1 ± 6.2 | | 100 | 582.8 ± 49.3 | 15.8 ± 0.33 |
| | | + | | 10 | 10° C. (0–16 d.); 1.5° C. (16–20 d.) | 32.2 ± 6.1 | 53.7† | 83 | 561.2 ± 41.7 | 17.0 ± 0.33† |
| | | + | + | 8 | 10° C. (0–16 d.); 1.5° C. (16–20 d.) | 31.5 ± 7.8 | 43.7† | 67 | 530.5 ± 47.5 | 17.0 ± 0.35† |

**Standard error of the mean.*
*†Significantly different from controls at room temperature.*

exhibit a maximal thyroid response, but those kept at room temperature and given ascorbate have a significantly ($p < 0.001$) higher epithelium than their controls at room temperature without ascorbate. This effect of DCA at low dosage level is particularly striking if we recall that the normal height of the thyroidal epithelium in rats of this age is around 8.0 $\mu$.

### C. Discussion

As we already noted in previous publications on this subject (7, 8), the administration of ascorbate to adrenalectomized rats receiving large doses of cortisone does not particularly change the growth rate, though it has some effect in reducing the extent of thymolysis and thyroid activation. The present results also confirm our previous observation (8) that large doses of DCA have effects similar to those of ascorbate administration as far as the same targets are concerned: thymus weight and thyroid activity.

However, from our last experiments (7) and this one, we gained evidence that the doses of cortical hormones used were too high. In order to see whether ascorbate administration still had some effect on resistance and adaptation to cold in animals with normal or subnormal corticoadrenal function, it was necessary to use smaller doses of cortical hormones or none at all in adrenalectomized rats. The doses used were suggested by the extensive studies of Selye and Bois (16).

From our results, it can be seen that ascorbate administration still retains some of its beneficial effects (better growth and survival) in adrenalectomized animals entirely deprived of cortical hormones. This strongly suggests that ascorbate might act, at least partly, through other systems than those directly influenced by changes in corticoadrenal function. This is further substantiated by the finding that, although the growth and survival are enhanced by the administration of minimal amounts of cortisone and DCA, ascorbate administration has the same relative effect on the growth and survival of animals exposed to cold as compared to their controls at room temperature. This again strongly indicates that ascorbate has some effects in animals exposed to cold which do not depend upon an increase in secretion of corticoadrenal hormones. It might be of interest to note that Booker *et al.* (5) have made a similar observation in their work on the effect of ascorbic acid on the survival of adrenalectomized mice exposed to cold without corticosteroid treatment. However, our results are in sharp contrast with those of Bacchus *et al.* (3), who reported a complete loss of action of ascorbic acid in adrenalectomized rats exposed to cold without cortical hormones, particularly on the survival. It seems to us that these results do not invalidate our findings, since the discrepancy may be explained through differences in experimental conditions.

It is true that adrenalectomized animals exposed to cold and given ascorbate are in a much better condition when they receive minimal amounts of cortical hormones. However, at the present time, it is difficult to see whether ascorbate has any direct effect, *in vivo*, on the peripheral action of cortical hormones as far as adaptation to cold is concerned. The cortical hormones might be

considered as having nothing but a "permissive" or "conditioning" action on the beneficial effects of ascorbate, provided the reactive ability of the organism towards cold stimulation is not exceeded.

As far as the thyroid effect of DCA is concerned, when it is administered alone at low dosage level, we would like to recall the work of Axelrad and Leblond (2). They found that an adequate supply of sodium chloride, as such, is indispensable for the development of goitrous thyroids under conditions of iodine deficiency in mice. The analogy between the present results, obtained with DCA treatment, and those found by Axelrad and Leblond using sodium chloride seems to be of a particular interest in the light of the recent observations of Salgado (14, 15) on the role of the thyroid gland in the production of cardiovascular diseases induced by DCA.

## II. Thyroidectomized Animals

These last results appeared sufficient to support the hypothesis of an extra-adrenal effect of ascorbate in animals exposed to cold, since our previous findings (7, 8, 9) had already suggested some relation between the thyroid and ascorbate.

### *1. Experimental*

Sixty-four male albino rats of the Wistar strain, weighing between 145 and 164 gm., were used in this experiment. Ten were kept as non-operated controls and the others were thyroidectomized at day 0 of the experiment. They were all fed the usual Purina diet, supplemented by calcium lactate 1% in the drinking water of the operated animals. The treatments were initiated 24 hr. after operation and maintained throughout according to the distribution indicated in Table III. The thyroxine was administered in one daily subcutaneous injection as thyroxine-Na (BDH) in a slightly alkaline solution. The ascorbate was administered orally as in the previous experiments. All the animals were kept at 24 $\pm$ 1.0° C. for the first seven days of the experiment; they were then transferred into a cold room at 14 $\pm$ 1.0° C. for another seven days, after which the remaining animals were sacrificed. The thymus and adrenals were weighed in the fresh state; the thyroid of the non-operated controls was fixed and prepared for epithelium measurements as described previously, and comparisons were made with the thyroids removed at the onset of the experiment.

### *2. Results (Table III)*

At this temperature of exposure, intact rats become readily acclimatized to cold. Their thyroids exhibit a definite and significant ($p < 0.001$) activation, as compared to the thyroid glands removed 14 days previously in the operated animals. They otherwise show no sign of alarm reaction, the adrenal and thymus weights being quite normal for rats of this age.

Thyroidectomized rats given ascorbate without thyroxine have a very low growth rate at room temperature and are almost all eliminated during exposure

TABLE III

EFFECTS OF ASCORBATE ADMINISTRATION AND THYROXINE IN THYROIDECTOMIZED RATS EXPOSED TO COLD

| Treatment | No. animals at autopsy | Growth in gm. 0–7th d. | Growth in gm. 7–14th d. | % survival | Thymus, fresh wt., mgm. | Adrenals, fresh wt., mgm. | Thyroid, height of epithelium, $\mu$ |
|---|---|---|---|---|---|---|---|
| Untreated, non-operated rats | 10 | 32.3 | 30.4 | 100 | 366.7 ± 14.22* | 27.8 ± 1.93* | 12.82 ± 0.36* |
| Thyroidectomized | | | | | | | |
| Ascorbate (150 mgm.) | 3 | 5.0 | −15.2 | 20 | 83.4 ± 9.17†,‡ | 32.6 ± 1.19† | |
| Thyroxine (10 $\mu$gm.) | 10 | 15.6 | 31.3 | 100 | 348.2 ± 17.34 | 29.7 ± 2.12 | |
| Thyroxine (3 $\mu$gm.) | 10 | 18.2 | 3.5 | 55 | 207.3 ± 23.68† | 35.9 ± 1.32† | 7.97 ± 0.18* at operation |
| Thyroxine (3 $\mu$gm.) + ascorbate (150 mgm.) | 10 | 19.1 | 21.7 | 91 | 316.2 ± 29.73 | 28.6 ± 2.06 | |

**Standard error of the mean.*
*†Significantly different from untreated, non-operated rats.*
*‡Significantly different from thyroxine (3 $\mu$gm.) treated animals.*

to cold. The surviving animals show a characteristic alarm reaction with loss of weight, thymus involution ($p < 0.001$), and adrenal enlargement ($p < 0.05$). Ascorbate administration shows no beneficial effects in the absence of adequate thyroid function replacement therapy.

In contrast, in the operated rats receiving 10 μgm. of thyroxine, exposure to cold does not seem to have any deleterious effects. This confirms our previous observations (7) as well as those of Leblond and Eartly (12).

In the thyroidectomized rats given only 3 μgm. of thyroxine, we observe the usual signs of an alarm reaction with a survival of only 55%. This again is a confirmation of the findings of Leblond and Eartly (12).

The most striking results were obtained in the thyroidectomized rats simultaneously treated with ascorbate and 3 μgm. of thyroxine. We have seen that, used separately, these treatments provide an entirely inadequate protection against exposure to cold. It is quite clear that ascorbate enhances the effect of a low dose of thyroxine in animals exposed to cold, which behave almost as intact animals or thyroidectomized animals receiving adequate amounts of thyroxine.

*3. Discussion*

In a previous paper (7), we discussed at some length the relations between the thyroid hormones and adrenaline. Since that time, other workers have accumulated evidence to show the role of epinephrine and nor-epinephrine on the thyroidal release of thyroid hormones (1, 13), as well as the importance of the combined effects of the thyroid hormones and those of the adrenal medulla in thermogenesis during exposure to cold (4, 6, 17, 19). It must also be noted that some relationship has been found (11) between the thyroid hormones and the metabolism of ascorbic acid. It is not surprising to find that ascorbate administration in animals exposed to cold seems to bear a definite relation to the thyroid hormones.

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## Résumé

Nous avons étudié les effets de l'administration d'ascorbate sur la résistance au froid et la fonction thyroïdienne de rats surrénaloprives traités à la cortisone et/ou à la DCA en doses variables, alors que chez le rat sans thyroïde traité ou non à la thyroxine, nous avons évalué la résistance au froid et la fonction de la cortico-surrénale.

Chez le rat sans surrénales exposé au froid et recevant de fortes doses de cortisone (2.5 mg.), l'administration d'ascorbate (150 mg.), ou de DCA

(2.5 mg.) augmente la survie, réduit le degré de thymolyse, et modère l'activation de la thyroïde. Chez le rat surrénalectomisé ne recevant ni cortisone ni DCA ou ne recevant que de faibles doses (0.4 et 0.1 mg.) de ces hormones, l'administration d'ascorbate conserve des effets bienfaisants: la croissance et la survie sont meilleures. Si la DCA est administrée seule à faible dose (0.1 mg.), c'est-à-dire non associée à la cortisone, l'ascorbate semble devenir nocif: la croissance et la survie des animaux exposés au froid sont diminuées, et ce phénomène s'accompagne d'une activation considérable de la thyroïde.

Chez le rat éthyroïdé exposé au froid, l'administration d'ascorbate (150 mg.) est sans effet en l'absence d'hormones thyroïdiennes, mais augmente l'efficacité de faibles doses (3 $\mu$g.) de thyroxine sur la croissance et la survie, supprimant en même temps certains signes typiques de la réaction d'alarme: la thymolyse et l'hypertrophie des surrénales.

Ces résultats semblent montrer que le rôle des hormones corticales dans la résistance au froid se limiterait à une action de support, et que l'effet bienfaisant de l'ascorbate serait plutôt relié à l'action des hormones de la thyroïde.

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# ETHYLENE METABOLISM IN TOMATO FRUIT

## I. RELATIONSHIP OF ETHYLENE EVOLUTION TO FRUIT RESPIRATION AND RIPENING[1]

By Mary S. Spencer


## Abstract

Ripening tomatoes were found to possess a climacteric peak in carbon dioxide production which was accompanied by evolution of ethylene. When the fruit was placed in oxygen, ripening and evolution of ethylene were accelerated in proportion to the increase in respiration. Peaks in carbon dioxide and ethylene evolution occurred not only when the fruit ripened, but also afterwards, during senescence. On the other hand, young, green fruit possessed a pronounced peak in carbon dioxide evolution without changes in color or texture or evolution of significant amounts of ethylene. Thus a series of waves in carbon dioxide production occurred as the tomato developed, ripened, and aged. Waves in ethylene evolution accompanied the carbon dioxide once the ripening process began. Evolution of large amounts of ethylene appeared to be associated with changes in the metabolic pathway that occurred with ripening, rather than being an accompaniment for increased respiration at any stage in the development of the fruit.


## Introduction

As most fruits ripen, they respire rapidly and give off ethylene. The sudden rise in respiratory rate associated with ripening is called the "climacteric", and is indicative of a transition stage in the development of the fruit between ontogeny and senescence.

Ethylene is not only produced by ripening fruit, but it also has marked physiological effects on fruit (16) and other parts of plants (1, 5) as well as on animals (7). Administration of minute amounts of ethylene to unripe (preclimacteric) fruit is used commercially to accelerate ripening. Although it hastens the onset of the climacteric, ethylene does not affect the progress of the climacteric once it is initiated (1).

The role of ethylene in fruit ripening is controversial. Hansen (11) postulated that ripening occurs when the concentration of ethylene in the fruit becomes sufficiently high. However, the fact that artificially supplied ethylene precipitates this change is not conclusive proof that natural ripening is initiated by ethylene produced by the fruit.

If ethylene does not cause the onset of the climacteric in normal ripening, the alternative is that it is merely a product of the ripening process, and not essential to it. Biale, Young, and Olmstead (3) obtained evidence for the latter view from one species of fruit, the mango, which ripened with a pronounced climacteric but without any evolution of ethylene. Nine other ripening fruits which they examined (sapote, pear, cherimoya, peach, papaya, feijoa, avocado, persimmon, pear) had marked carbon dioxide maxima which were accompanied by peaks in ethylene evolution. Similar behavior was

[1]*Manuscript received June 25, 1956.*
*Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta.*

found for apples (15) and bananas (14) by Nelson and for pears by Hansen (10). The mango is the only fruit that has been found to exhibit a climacteric without evolution of ethylene. No fruit is known which produces ethylene but does not have a climacteric (3).

As a first step in the clarification of the metabolic role of ethylene in fruit we decided to study the relationships among ethylene evolution, respiration, and ripening. Comparisons of amounts and times of occurrence of carbon dioxide and ethylene evolution were made as fruit ripened normally from the preclimacteric stage. Fruit was ripened in oxygen to increase the respiratory rate above normal, and the effect on ethylene evolution and ripening was determined.

Numerous experiments (1, 16) have shown that immediately before the climacteric both respiration and ethylene production are at a low level. Experiments were conducted with fruit in still earlier stages of development to determine whether ethylene was evolved when respiration, but not ripening, was taking place.

## Materials and Methods

### *Fruit*

Tomatoes (*Lycopersicon esculentum*) were chosen as subjects for experiment because of their commercial importance and their availability throughout the year. Most of the work was done with tomatoes of the Vitamold 121 variety, grown from seed in the University of Alberta greenhouses. Comparisons were made with commercial tomatoes of the Rutger and Marglobe varieties shipped from Texas and Mexico respectively to a local wholesaler. Tomatoes detached from the plant were used in all experiments.

Careful selection of tomatoes for maturity was made. Fully grown tomatoes were classified for ripeness according to the system of Heinze and Craft (12). Tomatoes in the "mature green" stage (full-size, wholly green except for a white star-shaped area at the stylar end of the fruit) constituted Class 1. Class 2 included tomatoes which showed "slight turning"; that is, a trace to one-quarter of the surface showed a yellow or red color. Class 3 ("medium turning") consisted of tomatoes with one-quarter to one-half of their surface a red color, while in Class 4 ("advanced turning") one-half to three-quarters of the surface was red. Class 5 consisted of "firm ripe" tomatoes with three-quarters to all of the surface red. After the tomatoes reached stage 5, the red color deepened and a gradual softening of the fruit ensued.

With tomatoes which had not yet reached the "mature green" stage, maturity was more difficult to determine. Gustafson (8), from experiments in which he tagged tomato flowers as they opened, found that the age of tomatoes was not a dependable indication of maturity. Doran, Holdsworth, and Rhodes (6) and Gustafson (9) showed that growth of a tomato involves first a phase of cell division (which lasts only about a week from setting), followed by a phase of cell enlargement. The latter phase persists until the

mature green stage is reached, after which the fruit undergoes little change in volume or weight, and begins to ripen. Therefore size was used as a basis of selection for experiments with young fruit: the tomatoes chosen were less than half as large as ripening fruit in the same vicinity on the same plant.

*Respiratory Chamber*

A stream of air was passed at constant rate, through a flowmeter, into a respiratory chamber which contained 2–4 kgm. fruit, and then out to an absorber for carbon dioxide or ethylene. The respiratory chamber consisted of a large glass jar equipped with desiccator plates (or masonite drilled with holes) to hold the tomatoes. A number of plates, separated and supported by appropriate lengths of glass rods, provided support for a large number of tomatoes and eliminated crushing of the lower layers. A lead plate and a rubber gasket sealed the jar at the top. The plate was fastened by long bolts to a heavy plywood base upon which the jar rested. For gas passage through the chamber two short lengths of copper tubing were soldered into holes bored in the lead plate. For the gas inlet a glass tube which reached to the bottom of the chamber was inserted inside one copper tube, and a seal made between the two tubes by rubber pressure tubing. The other copper tube was the gas outlet. The jar was wound with $\frac{3}{8}$ in. copper tubing through which cold tap water was circulated constantly to minimize the effects of variation in room temperature. (The temperature inside the sealed chamber was about 28° C.).

*Carbon Dioxide Determination*

Carbon dioxide was determined by Biale's and Shepherd's (2) modification of Truog's (21) method. Air from a compressed air tank was passed through a series of Drierite and ascarite U-tubes to free it of carbon dioxide, and then through a flowmeter at a rate of 350 ml./min. and into the respiratory chamber. The gases from the chamber were carried by the air stream into a tower tube that contained barium hydroxide, which absorbed the carbon dioxide. Excess barium hydroxide was determined by titration, and the amount of carbon dioxide absorbed was calculated. Duplicate or triplicate one hour determinations were run in succession each day. Maximum variation allowed for replicates was 5% and no variation in carbon dioxide evolved with the time of day was detected.

It was assumed that $CO_2$ evolution was an adequate measurement of respiratory rate for these experiments and that the amounts of gases evolved by the fruit were proportional to the amounts inside the fruit.

*Ethylene Determination*

Air from the compressed air tank was purified from unsaturated hydrocarbons by passage through a column of brominated, activated charcoal, according to the method described by Southwick (19) and Southwick and Smock (20). The purified air was passed through a flowmeter into the respiratory chamber at a rate of 300 ml./min.

Quantitative determinations of ethylene evolved by the fruit were made by the method of Young, Pratt, and Biale (24). Gas from the respiratory chamber was passed into mercuric perchlorate, where an ethylene–mercury complex was formed. The accumulated ethylene was later released from the complex by the addition of lithium chloride, and the volume of the gas was measured with a Warburg manometer. Total amounts of ethylene of 0.2 ml. could be determined with an accuracy of 5%, while higher accuracy was obtained, of course, with larger amounts. Minimum precision obtained (with the lowest total amounts of ethylene) was 12%.

Where both ethylene and carbon dioxide determinations were made on the same batch of fruit, respiration measurements were made over a period of 2 to 4 hr. each day while ethylene was absorbed for 20 to 22 hr. At least two complete runs were made for each variation in the experimental conditions.

*Biological Test for Ethylens*

Etiolated Alaska pea seedlings show a "triple response" to the presence of ethylene, even in minute quantites (18). The triple response involves (*a*) a change of negative geotropism to diageotropism, (*b*) increased growth in thickness, and (*c*) reduced rate of growth in length. Test sets of etiolated seedlings prepared by the method of Pratt and Biale (18) were placed inside the respiratory chamber and control seedlings were placed outside, and differences in growth patterns were observed.

*Preparation of Ethylene Derivatives*

The ethylene accumulated in mercuric perchlorate was released to form the ethylene dibromide and N,N′-diphenylethylenediamine derivatives, by the use of Pratt's (17) modification of the method of Young, Pratt, and Biale (23).

## Results and Discussion

*Tomatoes as Experimental Subjects*

Tomatoes were first tested for their suitability for a study of ethylene metabolism. Mature green tomatoes of the Vitamold 121 variety, grown in our own greenhouse, and two commercial varieties from the United States and Mexico, were found to exhibit a typical climacteric rise in respiration. The respiratory peak occurred in all varieties when the fruits were in a "medium turning" stage. By the time the fruits were firm ripe, respiration had decreased to a low level. This corresponds to the reports of other workers (9, 13) for detached tomatoes. Also, Walford (22) and Clendenning (4) demonstrated that tomato fruit ripening on the plant exhibited a similar behavior.

Tomatoes of all varieties were shown to evolve ethylene as they passed through the climacteric. Not only was the presence of ethylene indicated by the selective mercuric perchlorate absorption method used for its quantitative determination, but further evidence for its identity was obtained from the triple response of pea seedlings. (This expanded and confirmed the earlier

observations of Heinze and Craft (12), who had obtained positive biological tests indicative of ethylene in the gaseous exudate from ripening tomatoes.) The N,N′-diphenylethylenediamine derivative was found to have a melting point of 65°–67° as compared with a melting point of 65°–66° for the Eastman white label product. The melting point was not lowered when the two were mixed.

Thus there remained no doubt that ethylene was produced by tomatoes, and that ripening tomatoes exhibited a typical "climacteric" and that they were suitable for studies of ethylene metabolism.

*Comparisons of Carbon Dioxide and Ethylene Evolution*

*Mature Green Tomatoes Ripened in Air*

Information on the causal relationship between ethylene production and the onset of the climacteric rise in respiration was sought through sequential measurements of the carbon dioxide and ethylene evolved by mature green tomatoes as they ripened (Fig. 1). The initial decrease in respiration is typical of mature green tomatoes when they are either attached to (4) or detached from (9) the plant, and this is probably related to the cessation of growth. Immediately before the climacteric rise both the ethylene and carbon dioxide were at a minimum. The ethylene began to increase shortly before the carbon dioxide did, which is consistent with, but not proof of, Hansen's (11) hypothesis that ripening occurs when the concentration of ethylene becomes sufficiently high.

The peak in carbon dioxide production preceded the peak in ethylene evolution. Ethylene was evolved in much smaller quantities than carbon

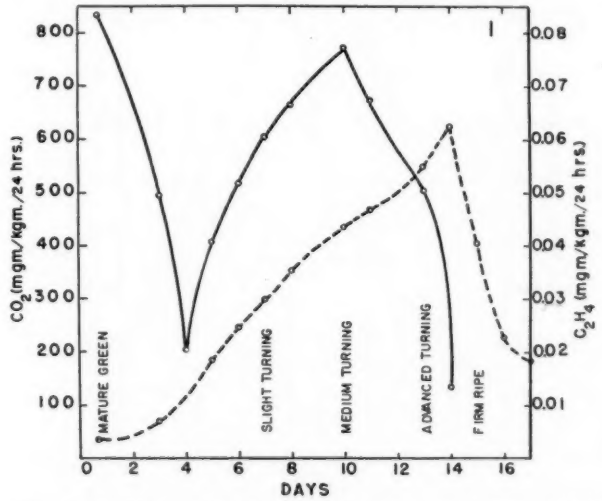


FIG. 1. Carbon dioxide and ethylene production of tomatoes (Vitamold 121 variety) ripened from the mature green stage in air. (Solid line $CO_2$, broken line $C_2H_4$.)

dioxide. From the 4th to the 10th day carbon dioxide was produced at the rate of 1.9 mgm. (43 $\mu$M.)/kgm./24 hr., while ethylene was given off at a rate of 1 mgm. (36 $\mu$M.)/kgm./24 hr. From the 8th to the 12th day, the rate of ethylene production was 0.8 mgm./kgm./24 hr. That is, ethylene evolution did not parallel carbon dioxide evolution in every respect, although it was related to it.

*Mature Green Tomatoes in Oxygen*

The extent of the connection between ethylene production and respiration was investigated further by experiments in which mature green tomatoes were ripened in oxygen rather than in air. The results are recorded in Fig. 2. Valid comparisons could be made with the results recorded in Fig. 1, for in both experiments tomatoes of the same age and variety (Vitamold 121), which were grown under similar conditions in the greenhouse, were used. As would be expected, respiration reached much higher levels in oxygen than in air. It was interesting that the ethylene evolution was proportionately high, with production of both gases in oxygen about double that in air. In oxygen the fruit passed very quickly through the climacteric and the maximum in ethylene evolution. Also, the ripening process took place much more rapidly in oxygen than in air, as tomatoes passed from the mature green to the advanced turning stage in 4 days rather than 13. The peaks in gas evolution occurred at the same stage of ripeness in oxygen as in air. The fact that

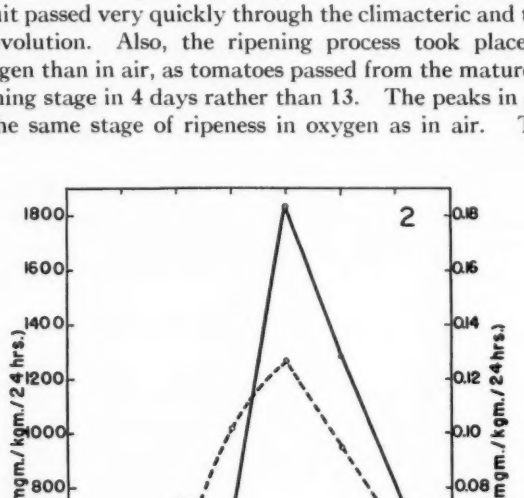


FIG. 2. Carbon dioxide and ethylene production of tomatoes (Vitamold 121 variety) ripened from the mature green stage in oxygen. (Solid line $CO_2$, broken line $C_2H_4$.)

ethylene evolution and ripening were accelerated in proportion to the increase in respiration when fruit was placed in oxygen showed an extremely close relationship existed among the three (respiration, ripening, and ethylene evolution).

*Postclimacteric Tomatoes*

Both in air and in oxygen, respiration and ethylene evolution declined to low levels shortly after the climacteric peaks had been passed (Figs. 1 and 2). Collection of gases was continued beyond this stage for several batches of tomatoes. The tomatoes were initially sound and showed no deterioration from mold or rot during the extended period.

The results are recorded in Figs. 3 and 4. Further rises and falls in carbon dioxide production and ethylene evolution occurred after the first climacteric peak. Although the magnitudes of the peaks gradually decreased, they were still several times greater than could be accounted for by analytical errors. Total amounts of gases given off were of sufficient size to allow reasonable accuracy of determination, even during periods of minimum evolution. For mature green tomatoes ripened in air (Fig. 3), on the 20th day the quantity of ethylene collected was 0.06 ml.; for mature green tomatoes ripened in oxygen (Fig. 4) 0.19 ml. of ethylene was collected on the seventh day. It did not seem likely that the additional peaks were due to losses during collections of the gases, for at least two separate collections were made for each carbon dioxide determination. Also, ethylene and carbon dioxide collections were made separately, and the shapes of the curves for the two gases were very similar. Since the peaks were obtained at similar intervals in several different runs, it did not seem probable that they were due to temperature variations.

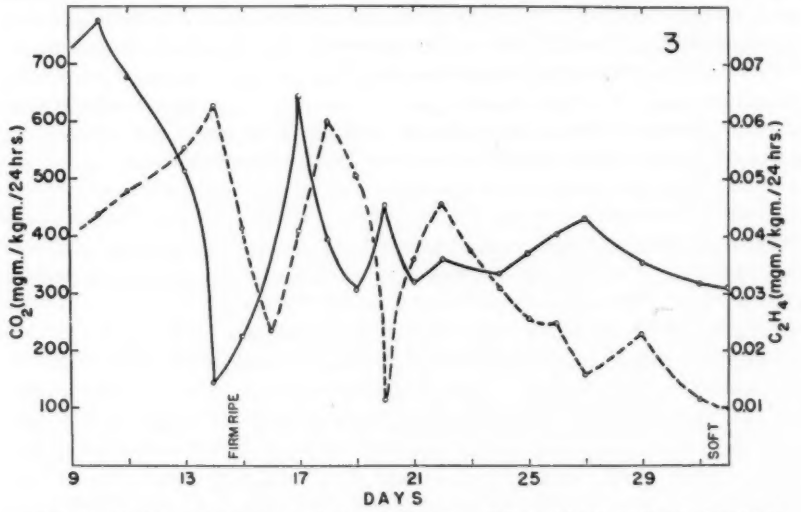


FIG. 3. Postclimacteric gas evolution of tomatoes (Vitamold 121 variety) ripened in air. (Solid line $CO_2$, broken line $C_2H_4$.)

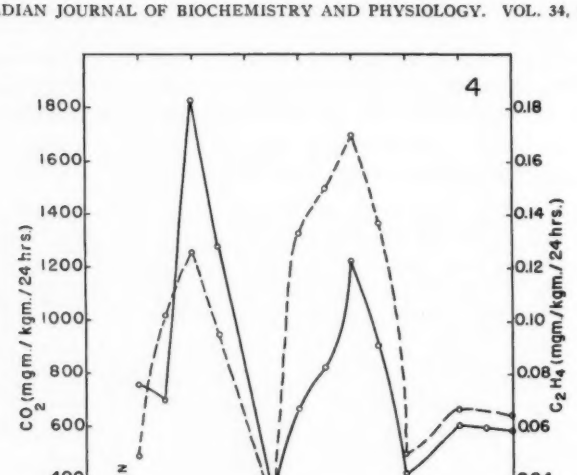


FIG. 4. Postclimacteric gas evolution of tomatoes (Vitamold 121 variety) ripened in oxygen. (Solid line $CO_2$, broken line $C_2H_4$.)

In air the ethylene peaks came after the maxima in carbon dioxide production, while in oxygen the peaks appeared to coincide. In oxygen, where the maxima were reached and passed very quickly, the gas collection intervals were probably too long for the exact sequence of peaks to be distinguished.

Thus, although previously considered unique for the "ripening" stage, the climacteric peak in carbon dioxide and its accompanying increase in ethylene evolution was just one of several such peaks. The production of gases in waves continued, though with lesser intensity, during senescence.

*Young Tomatoes*

When ethylene and carbon dioxide had been shown to be evolved in waves both through the ripening process and through senescence, the obvious question was whether young fruit had a similar behavior.

Results from experiments with young tomatoes are presented in Fig. 5. Respiration reached higher levels with the small tomatoes than with full-size tomatoes of the same variety. This is consistent with the work of Melvin (13).

A pronounced peak in carbon dioxide evolution occurred while the fruit was still green. This peak was higher than the climacteric and senescent peaks. On the other hand, while ethylene evolution increased slightly with this increase in carbon dioxide, it remained at relatively low levels. A large increase in ethylene evolution did not occur until the climacteric. Thus it

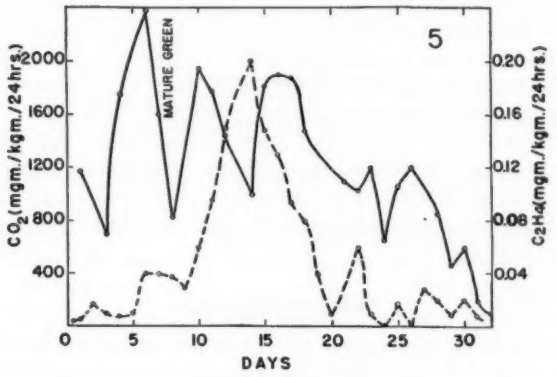


FIG. 5. Carbon dioxide and ethylene production, in air, of tomatoes (Vitamold 121 variety) picked when young. (Solid line $CO_2$, broken line $C_2H_4$.)

was clear that evolution of large amounts of ethylene was characteristic of the ripening and senescent processes, and did not automatically follow an increase in carbon dioxide production at other stages in the development of the fruit.

The physiological effect of removal of the young fruit from the plant may be questioned. Clendenning (4) showed that mature green tomatoes isolated from the plant exhibited the same respiratory behavior as those fruits left attached to the plant. Young fruits, in contrast to mature green fruits, are in a stage of active growth caused by increase in cell size (since cell division has ceased) (6, 9). This growth, of course, ceases when the fruits are picked, and therefore the behavior of detached young fruit may be different from young fruit on the plant. Whether or not this is so does not alter the fact that a respiratory peak was obtained without significant ethylene evolution and without ripening.

After the first peak in respiration of the young, green fruits, ripening and the climacteric and senescent peaks in carbon dioxide and ethylene evolution occurred in the same way as for fruits picked when full size (Fig. 5). However, the rates of ripening varied much more for the young tomatoes than for tomatoes picked when in the mature green stage. This was probably a result of considerable age variation in the young tomatoes, whereas tomatoes in the mature green stage were in a definite, narrow range of maturity. The uneven ripening of the small tomatoes is reflected in the two peaks in carbon dioxide production, at 10 and 16 days. By the 14th day 29% of the tomatoes were in stage 5, 23% in stage 4, 12% in stage 3, and 36% in stage 1. By the 18th day 91% of the tomatoes were in stage 5. The peak in ethylene production, which normally occurs at a slightly later stage in maturity than the peak in carbon dioxide production, straddled the two carbon dioxide peaks. Thus the period from 8 to 18 days on the curves reflects the transition of the tomatoes, at various rates, through the climacteric and ripening processes.

Young tomatoes as well as full-size tomatoes evolved some ethylene at each stage in their development. Recent evidence (17) indicates that ethylene is produced by other parts of plants as well as fruits. It seems likely that all respiring plant tissues produce some ethylene. The amount produced is regulated by factors which are unknown at present.

In these experiments with young fruit an initial rise and fall in carbon dioxide production similar to that of the climacteric occurred without concurrent ripening and ethylene production. Apparently evolution of large amounts of ethylene was associated with changes in the metabolic pathway that occurred with ripening.

## Acknowledgments

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## NOTE

### A MANNAN PRODUCED BY BACILLUS POLYMYXA[1]

By D. Murphy, C. T. Bishop, and G. A. Adams

Polysaccharides which contain predominately D-mannose are widely distributed in nature and have been found in ivory nuts (2), orchid tubers (14), red algae (8), yeast (6, 5), and fungi (7). The alkali-soluble polysaccharides of some woods also contain D-mannose but a pure mannan has not yet been isolated from this source (14). The mannans from these various sources were all joined by $\beta$ 1 —> 4 glycosidic linkages except those isolated from yeast, which were shown to have a highly branched structure with 1 —> 6, 1 —> 2, and 1 —> 3 linkages. Recently Orlova (10) has isolated a mannan from diptheria bacteria and described some of its properties (11). This appears to be the first report of a bacterial mannan. We shall report here the preparation and some properties of another bacterial mannan, isolated in these laboratories from *Bacillus polymyxa*.

The polysaccharide was produced by the organism *B. polymyxa* C3 (2)* grown on a synthetic medium as described previously (9). When the fermentation was complete, the culture medium was centrifuged and concentrated to one-third its original volume. The various polysaccharides were separated by fractional precipitation with ethanol (95%) (9). The crude mannan was recovered in 1% yield (based on the sugar in the medium) as the first precipitate formed by addition of 1200 ml. of ethanol to 2 l. of the concentrated medium. This material had an ash content of 12.61% and a nitrogen content (Kjeldahl) of 1.59% (on ash-free basis). Dialysis against 0.1 *N* hydrochloric acid for 72 hr. lowered the ash content to 1.40% while three treatments with tertiary amyl alcohol and chloroform (13) lowered the nitrogen content to 0.19%. The product was now a white powder readily soluble in water. Estimation of the number-average molecular weight by the hypoiodite method (3) gave a value of 4620. Hydrolysis with 2 *N* $H_2SO_4$ at 100° C. for 10 hr. and chromatography of the hydrolyzate showed the presence of mannose along with small amounts of glucose, galactose, and uronic acid.

Purification of the polysaccharide was attempted by fractionation of its acetate from acetone solution with petroleum ether. Five fractions were recovered which differed only slightly from each other in acetyl content and specific rotation. Purification of the material by copper complexing using the method of Angell and Norris (1) yielded a product with a mannose content

[1]*Issued as N.R.C. No. 4091.*

**From the culture collection of the Division of Applied Biology, National Research Council of Canada, Ottawa.*

of 95.2%, the remainder being glucose, galactose, and uronic acid. An additional copper complexing treatment of the mannan yielded a product which contained 98% mannose and a trace of glucose. Electrophoretic examination of the hydrolyzate in $M/40$ phosphate buffer (pH 7.2) at 30 volts per centimeter showed the presence of an acidic material which was neither glucuronic acid nor galacturonic acid. The purified mannan, recovered from aqueous solution by freeze drying, was a crisp white solid readily soluble in water. Reducing power was determined by hypoiodite oxidation in phosphate buffer at pH 11.9 (4) and the results indicated a repeating unit having approximately 34 mannose residues.

The mannose in the polysaccharide was isolated and identified as follows: purified mannan (100 mgm.) was hydrolyzed with $N$ $H_2SO_4$ (10 ml.) at 100° C. and the initial specific rotation +76.8° changed to +24° (constant value) in eight hours. The acid hydrolyzate was neutralized, clarified with charcoal, and the sirup treated with methanol: isopropanol (1 : 2 v/v) to remove impurities of higher molecular weight. The purified mannose (75 mgm.) was crystallized from acetic acid solution and after recrystallization had a m.p. 130° C. and $[\alpha]_D^{26}$ + 14.8° ($c$, 1.5 in water) in good agreement with reported values (6). Treatment of the mannose sirup with phenylhydrazine yielded crystalline mannose phenylhydrazone m.p. 198–199° C. (unchanged on admixture with an authentic sample.)

The mannan was acetylated by acetic anhydride in formamide and pyridine solution. The triacetate was a white powder soluble in acetone, chloroform, pyridine; insoluble in water; acetyl content 44.0%, $[\alpha]_D^{26}$ + 66.6° ($c$, 0.73 in chloroform). Drying of the acetate from chloroform solution yielded a brittle film.

Periodate oxidation of the mannan by the manometric method (12) was complete in 45 hr. with a consumption of 1.14 M. of periodate per mole of anhydro-D-mannose and a production of 0.46 M. of formic acid per sugar residue. These results could arise from a straight chain mannan molecule having an equal number of 1 —> 3 and 1 —> 6 linkages or from a highly branched mannan containing 1 —> 2 or 1 —> 4 linkages. Some branching was indicated by the formation of a small amount of mannose after hydrolysis of the oxidized mannan. However the quantity obtained was so small (3%) that the 1 —> 3, 1 —> 6 linked type of structure is favored.

The properties of this mannan resemble more closely those of the diptheria mannan (11) than any of the others described (2, 8, 5, 7).

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# CONTENTS OF VOLUME 34

**Abe, T.** See Seegers, W. H., 270.

**Adams, G. A.** See Murphy, D., 1271.

**Adie, P. A.** The effect of the sarinase levels of liver on the survival of rabbits injected with sarin, 654.

The purification of sarinase from bovine plasma, 1091.

**Adie, P. A., Hoskin, F. C. G.,** and **Trick, G. S.** Kinetics of the enzymatic hydrolysis of sarin, 80.

**Alivisatos, S. G. A., Kashket, S.,** and **Denstedt, O. F.** The metabolism of the erythrocyte. IX. Diphosphopyridine nucleotidase of erythrocytes, 46.

**Allard, C., de Lamirande, G., Weber, G.,** and **Cantero, A.** Effect of cortisone on the intracellular distribution of phosphatases and ribonucleases in rat liver, 170.

**Altermatt, H. A.** and **Neish, A. C.** The biosynthesis of cell wall carbohydrates. III. Further studies on formation of cellulose and xylan from labeled monosaccharides in wheat plants, 405.

**Annau, E.** Spectrophotometric analysis of proteins by a selective filter technique, 236.

**Archibald, J.** See Markowitz, J., 422.

**Auer, J.** The structural basis of some corticodiencephalic relations, 372.

**Baer, E.** The synthesis of glycerolphosphatides, 288.

**Baker, D. G.** and **Sellers, E. A.** Electrolyte metabolism in the rat exposed to ionizing radiation, 835.

**Baker, E.** See Kovach, J. C., 883.

**Bandy, H. E.** See Southcott, C. M., 146, 913.

**Barker, A. D.** See Southcott, C. M., 146.

**Barr, M. L.** Discussion of paper by Auer, J. (Symposium on Neurophysiology), 379.

**Bayley, S. T.** See Webber, R. V., 993.

**Bélanger, L.-F.** See Crevier, M., 869.

**Bencosme, S. A.** and **Mariz, S.** Regional differences in the glucagon content of pancreas from alloxanized dogs, 779.

**Benoiton, L.** and **Bouthillier, L. P.** The metabolism of $\gamma$-hydroxyglutamic-$\alpha$-$C^{14}$ acid in the intact rat, 661.

**Berry, J. F.** Discussion of paper by Carter, H. E., Galanos, D. S., and Fujino, Y. (Symposium on Phospholipids), 331.

**Best, C. H.** See Young, R. J., 713.

**Beveridge, J. M. R.** The function of phospholipids, 361.

**Beveridge, J. M. R., Connell, W. F.,** and **Mayer, G. A.** Dietary factors affecting the level of plasma cholesterol in humans: The role of fat, 441.

**Beznák, A. B. L.** *With the technical assistance of* **Robineau, I.** Changes in the electrocardiogram and heart choline ester content of thiamine-deficient and pair-fed rats, 845.

**Beznák, M.** Hemodynamic changes following aortic constriction in normal and in hypophysectomized rats, 791.

**Bilinski, E.** See Dulaney, E. L., 1195.

**Birmingham, M. K.** See Schönbaum, E., 527.

**Bishop, C. T.** See Murphy, D., 1271.

**Blackwood, A. C.** See Spencer, J. F. T., 495.

**Boothroyd, B., Thorn, J. A.,** and **Haskins, R. H.** Biochemistry of the Ustilaginales. XII. Characterization of extracellular glycolipids produced by *Ustilago* sp., 10.

**Bouthillier, L. P.** See Benoiton, L., 661; Goldner, M., 116; Kraml, M., 783; Letellier, G., 1123.

**Boyd, E. M.** and **Tikkala, A. O.** Lipid and water levels in the kidneys of albino rats bearing Walker carcinoma, 256, 259.

**Brown, S. A.** and **Neish, A. C.** Studies of lignin biosynthesis using isotopic carbon. V. Comparative studies on different plant species, 769.

**Burgen, A. S. V.** A theoretical treatment of glucose reabsorption in the kidney, 466.

**Burns, B. D.** The electrophysiological approach to the problem of learning, 380.

The cause of reflex afterdischarge in the frog's spinal cord, 456.

**Buskirk, E. R.** See Iampietro, P. F., 721.

**Butler, G. C.** Discussion of paper by Kennedy, E. P. (Symposium on Phospholipids), 347.

**Cantero, A.** See Allard, C., 170.

**Carroll, K. K.** and **Noble, R. L.** Erucic acid and cholesterol excretion in the rat, 981.

**Carruthers, G. F.** and **Gowdey, C. W.** The effect of chlorpromazine given with reinfusion on the mortality rate from standardized hemorrhagic shock in the rat, 217.

**Carter, H. E., Galanos, D. S.,** and **Fujino, Y.** Chemistry of the sphingolipides, 320.

**Chefurka, W.** and **Smallman, B. N.** The occurrence of acetylcholine in the housefly, *Musca domestica* L., 731.

**Cho, M. H.** and **Jaques, L. B.** Heparinase. III. Preparation and properties of the enzyme, 799.

**Cleghorn, R. A.** Pharmacological and endocrinological influences on mentation and emotions, 390.
See also Murphy, C. W., 534.

**Collier, H. B.** See Solvonuk, P. F., 481.

**Common, R. H.** See Wright, L. A., 817.

**Connell, W. F.** See Beveridge, J. M. R., 441.

**Cook, F. S.** Adaptation of the carbon dioxide yield in the ninhydrin reaction to the study of transamination in plant tissues, 15.

**Crevier, M.** et **Bélanger, L.-F.** Étude quantitative de l'activité cholinestérasique de la plaque motrice par voie d'histophotométrie, 869.

**Darrach, M.** See Newsom, S. E., 1; Southcott, C. M., 146, 913.

**Deb, C.** and **Hart, J. S.** Hematological and body fluid adjustments during acclimation to a cold environment, 959.

**DeLuca, H. A.** See Gray, D., 763; Kline, D. L., 429.

**Denstedt, O. F.** Discussion of paper by Beveridge, J. M. R. (Symposium on Phospholipids), 370.
See also Alivisatos, S. G. A., 46; Malkin, A., 121, 130, 141; Rubinstein, D., 61, 222, 927.

**DesMarais, A.** Further studies on thyroid – adrenal – ascorbic acid relations in animals exposed to cold, 1251.

**Downie, H. G.** The effect of aureomycin on the production of hemorrhagic shock in the rat, 1119.

**Drake, C. G., Seguin, J. J.,** and **Stavraky, G. W.** The effects of convulsant agents on partially isolated regions of the central nervous system, 689.

**Duckert, F.** See Penner, J. A., 1199.

**Dulaney, E. L., Bilinski, E.,** and **McConnell, W. B.** Extracellular organic nitrogen in *Ustilago maydis* fermentation broths, 1195.

**Elliott, K. A. C.** See Pappius, H. M., 1007, 1053.

**Etebar, H.** See Kovach, J. C., 883.

**Fairbairn, D.** The muscle and integument lipids in female *Ascaris lumbricoides*, 39.

**Fenichel, R. L.** See Seegers, W. H., 270.

**Ferguson, J. K. W.** Discussion of paper by Cleghorn, R. A. (Symposium on Neurophysiology), 404.
See also McColl, J. D., 903.

**Ferguson, M. H., Naimark, A.,** and **Hildes, J. A.** Parotid secretion of iodide, 683.

**Fisher, K. C.** See Manery, J. F., 893.

**Fisher, L. M., Millar, G. J.,** and **Jaques, L. B.** The effect of oral and intravenous administration of vitamins K on the prothrombin and proconvertin levels of cholecystnephrostomized dogs, 1039.

**Florey, E.** The action of factor I in certain invertebrate organs, 669.

**Folch, J.** and **LeBaron, F. N.** The chemistry of the phosphoinositides, 305.

**Fregly, M. J.** See Iampietro, P. F., 721.

**Friedman, C. L.** See Friedman, S. M., 158, 475.

**Friedman, S. M., Radcliffe, R. W., Turpin, J. E. H.,** and **Friedman, C. L.** "Reflex" anuria in the dog, 158.

**Friedman, S. M., Webber, W. A.,** and **Friedman, C. L.** The effect of pitressin on the renal response to salt loading in the rat, 475.

**Fujino, Y.** See Carter, H. E., 320.

**Galanos, D. S.** See Carter, H. E., 320.

**Gandossi, S. K.** See Southcott, C. M., 146.

**Genest, J.** See Nowaczynski, W. J., 1023.

**Genest, K.** See Kalow, W., 637.

**Goldner, M.** and **Bouthillier, L. P.** A study of the effect of antihistamines on the metabolism of histamine in the intact rat, 116.

**Gourley, D. R. H.** See Manery, J. F., 893.

**Gowdey, C. W.** See Carruthers, G. F., 217; Spoerel, W. E. G. A., 747.

**Gowdey, C. W., Pearce, J. W.,** and **Stevenson, J. A. F.** Effect of tolazoline in hemorrhagic hypotension in dogs, 951.

**Gözsy, B.** See Kátó, L., 580.

**Gözsy, B.** and **Kátó, L.** Studies on the effects of phagocytic stimulation on microbial disease. V. Stimulation of phagocytic activity of monocytes against tubercle bacilli, strain BCG, 571.

**Gray, D. E.** and **DeLuca, H. A.** The effect of insulin administration on arteriovenous glucose differences in the alloxan-diabetic dog, 763.

**Hart, J. S.** See Deb, C., 959.

**Hart, J. S.** and **Heroux, O.** Utilization of body reserves during exposure of mice to low temperatures, 414.

**Haskins, R. H.** See Boothroyd, B., 10.

**Heagy, F. C.** See Nicholls, D., 543.

**Heroux, O.** See Hart, J. S., 414.

**Hildes, J. A.** See Ferguson, M. H., 683.

**Höhn, E. O.** Seasonal recrudescence of the thymus in adult birds, 90.

**Hokin, L. E.** and **Hokin, M. R.** Metabolism of phospholipids *in vitro*, 349.

**Hokin, M. R.** See Hokin, L. E., 349.

**Hoogland, P. L.** See Truscott, B., 191.

**Hoskin, F. C. G.** The enzymatic hydrolysis products of sarin, 75.

Some observations concerning the biochemical inertness of methylphosphonic and isopropyl methylphosphonic acids, 743.

See also Adie, P. A., 80.

**Hurst, R. O.** The degradation of desoxyribonucleic acid during alkaline hydrolysis, 265.

**Iampietro, P. F., Fregly, M. J.,** and **Buskirk, E. R.** Maintenance of body temperature of restrained adrenalectomized rats exposed to cold: Effect of adrenal cortical hormones, 721.

**Jaques, L. B.** See Cho, M. H., 799; Fisher, L. M., 1039; Taylor, J. D., 1143.

**Johnson, S. A.** See Penner, J. A., 1199; Seegers, W. H., 887.

**Jones, R. N.** See Nowaczynski, W. J., 1023.

**Kalow, W., Genest, K.,** and **Staron, N.** Kinetic studies on the hydrolysis of benzoylcholine by human serum cholinesterase, 637.

**Kaplan, J. G.** and **Paik, Woon-Ki.** The alteration of intracellular enzymes. IV. Kinetics of catalase alteration induced by chemical agents, 25.

**Kashket, S.** See Alivisatos, S. G. A., 46; Rubinstein, D., 61.

**Kates, M.** Hydrolysis of glycerolphosphatides by plastid phosphatidase, C, 967.

**Kátó, L.** See Gözsy, B., 571.

**Kátó, L.** and **Gözsy, B.** Studies on the effects of phagocytic stimulation on microbial disease. VI. Some factors decreasing phagocytic activity of monocytes against tubercle bacilli, strain BCG, 580.

**Kennedy, E. P.** The biological synthesis of phospholipids, 334.

**Kline, D. L.** and **DeLuca, H. A.** Phospholipid metabolism in liver slices. Labelling of phospholipids with acetate-1-$C^{14}$, 429.

**Koiw, E.** See Nowaczynski, W. J., 1023.

**Kovach, J. C., Baker, E.,** and **Etebar, H.** Residual air determination, 883.

**Kovacs, E.** Comparative biochemical studies on normal and on poliomyelitis infected tissue cultures. I. Observations on synthetic nutrient mixtures incubated with tissue cultures of normal kidney, 273.

Comparative biochemical studies on normal and on poliomyelitis infected tissue cultures. II. Investigation of various enzyme systems in homogenates of kidney tissue cultures of normal rhesus monkeys, 600.

Comparative biochemical studies on normal and on poliomyelitis infected tissue cultures. III. Enzyme assays on homogenates of surviving normal rhesus kidney. Effect of synthetic nutrient mixtures, 619.

**Kovacs, L.** and **Tuba, J.** A note on the energy of activation of the amylase in various human body fluids, 6.

**Kowalewski, K., Norvell, S. T., Jr.,** and **MacKenzie, W. C.** The gastric endocrine and exocrine response to histamine in dogs and effect of passage of blood through the gastric and hepatic vessels on its pepsinogen content, 244.

**Kraml, M.** and **Bouthillier, L. P.** Further studies on the metabolism of urocanic-α-$C^{14}$ acid, 783.

**Lagnado, J. R.** and **Sourkes, T. L.** Inhibition of amine oxidase by metal ions and by sulphydryl compounds, 1185.

The enzymatic reduction of tetrazolium salts by amines, 1095.

**de Lamirande, G.** See Allard, C., 170.

**LeBaron, F. N.** See Folch, J., 305.

**Letellier, G.** and **Bouthillier, L. P.** The formation of 2-pyrrolecarboxylic acid from hydroxy-D- and allohydroxy-D-proline, 1123.

**Linford, J. H.** Experiments on the use of silicic acid for steroid chromatography, 1153.

**Lucas, C. C.** Discussion of paper by Folch, J. and LeBaron, F. N. (Symposium on Phospholipids), 318.

See also Young, R. J., 713.

**McArthur, C. S.** Discussion of paper by Baer, E. (Symposium on Phospholipids), 303.

**McColl, J. D., Parker, J. M.,** and **Ferguson, J. K. W.** Experimental evaluation of diuretics in the rat, 903.

**McConnell, W. B.** See Dulaney, E. L., 1195.

**McConnell, W. B.** and **Ramachandran, L. K.** Acetate metabolism of maturing wheat plants, 180.

**McIntosh, H.** See Southcott, C. M., 146.

**MacIntosh, M.** See Moya, F., 563.

**MacKenzie, W. C.** See Kowalewski, K., 244.

**Maclachlan, G. A.** and **Waygood, E. R.** Kinetics of the enzymically-catalyzed oxidation of indoleacetic acid, 1233.

**McRae, S. C.** See Solvonuk, P. F., 481.

**Main, A. R.** The role of A-esterase in the acute toxicity of paraoxon, TEPP, and parathion, 197.

**Malkin, A.** and **Denstedt, O. F.** The metabolism of the erythrocyte. X. The inorganic pyrophosphatase of the erythrocyte, 121.

The metabolism of the erythrocyte. XI. Synthesis of diphosphopyridine nucleotide in the erythrocyte, 130.

The metabolism of the erythrocyte. XII. Diphosphopyridine nucleotide nucleosidase of the rabbit erythrocyte, 141.

**Manery, J. F., Gourley, D. R. H.,** and **Fisher, K. C.** The potassium uptake and rate of oxygen consumption of isolated frog skeletal muscle in the presence of insulin and lactate, 893.

**Mariz, S.** See Bencosme, S. A., 779.

**Markowitz, J.** and **Archibald, J.** Transbuccal hypophysectomy in the dog, 422.

**Matheson, A. T.** See Tamboline, B., 815.

**Maw, W. A.** See Wright, L. A., 817.

**Mayer, G. A.** See Beveridge, J. M. R., 441.

**Merler, E.** See Whitaker, D. R., 83.

**Migicovsky, B. B.** See Wood, J. D., 861.

**Millar, G. J.** See Fisher, L. M., 1039; Taylor, J. D., 1143.

**Monkhouse, F. C.** The extractable heparin in different animal tissue, 757.

**Mortimer, D. C.** See Towers, G. H. N., 511.

**Moya, F., Szerb, J. C.,** and **MacIntosh, M.** Identification of a hyperglycemic factor in urine, 563.

**Murphy, C. W.** and **Cleghorn, R. A.** Study of adrenocortical physiology in jet flying, 534.

**Murphy, D., Bishop, C. T.,** and **Adams, G. A.** A mannan produced by *Bacillus polymyxa*, 1271.

**Naimark, A.** See Ferguson, M. H., 683.

**Neish, A. C.** See Altermatt, H. A., 405; Brown, S. A., 769; Spencer, J. F. T., 495.

**Newsom, S. E.** See Southcott, C. M., 913.

**Newsom, S. E.** and **Darrach, M.** A comparison of the effects of certain corticosteroids and related compounds on the production of circulating hemolytic antibodies in the mouse. I. Structural variations at C-11 and C-17, 1.

**Nicholls, D.** Effect of ascorbic acid on the phosphorus metabolism of the adrenal gland, 919.

**Nicholls, D., Heagy, F. C.,** and **Rossiter, R. J.** Phosphorus metabolism of the adrenal gland of the rat. Effect of exposure to a cold environment for eight days on the amounts and $P^{32}$-labelling of phospholipid and ribonucleic acid, 543.

**Noble, R. L.** See Carroll, K. K., 981.

**Norvell, S. T., Jr.** See Kowalewski, K., 244.

**Nowaczynski, W. J.** and **Steyermark, P. R.** Further studies on the absorption spectra of steroids in "100%" phosphoric acid, 592.

**Nowaczynski, W. J., Steyermark, P. R., Koiw, E., Genest, J.,** and **Jones, R. N.** Detailed study of a purified urinary aldosterone fraction, 1023.

**O'Brien, R. D.** Activation of schradan by mammalian tissue homogenates, 1131.

**Ottolenghi, P.** See Rubinstein, D., 222.

**Packham, M. A.** The absorption of labelled glucose, sodium glucuronate, and glucurone from the gastrointestinal tract of the rat, 587.

**Paik, Woon-Ki.** See Kaplan, J. G., 25.

**Pappius, H. M.** and **Elliott, K. A. C.** Water distribution in incubated slices of brain and other tissues, 1007.

Factors affecting the potassium content of incubated brain slices, 1053.

**Parker, J. M.** See McColl, J. D., 903.

**Patterson, J. M.** See Young, R. J., 713.

**Pearce, J. W.** Discussion of paper by Burns, B. D. (Symposium on Neurophysiology), 388. See also Gowdey, C. W., 951.

**Penner, J. A.** See Seegers, W. H., 887.

**Penner, J. A., Duckert, F., Johnson, S. A.,** and **Seegers, W. H.** Conversion of prothrombin to autoprothrombin II, 1199.

**Peretz, S.** See Polglase, W. J., 558.

**Polglase, W. J.** The effect of dihydrostreptomycin on the formation of *beta*-galactosidase by *Escherichia coli*, 554.

**Polglase, W. J., Peretz, S.,** and **Roote, S. M.** Adaptive enzyme formation by dihydrostreptomycin-dependent *Escherichia coli*, 558.

**Radcliffe, R. W.** See Friedman, S. M., 158.

**Ramachandran, L. K.** See McConnell, W. B., 180.

**Rixon, R. H.** and **Stevenson, J. A. F.** The water and electrolyte metabolism of rat diaphragm *in vitro*, 1069.

**Ronwin, E.** Enzymatic properties of bovine plasmin preparations; evidence for similarity to but non-identity with trypsin, 1169.

**Roote, S. M.** See Polglase, W. J., 558.

**Rossiter, R. J.** Discussion of paper by Hokin, L. E. and Hokin, M. R. (Symposium on Phospholipids), 358.

See also Nicholls, D., 543.

**Rubinstein, D.** and **Denstedt, O. F.** The metabolism of the erythrocyte. XIV. Metabolism of nucleosides by the erythrocyte, 927.

**Rubinstein, D., Kashket, S.,** and **Denstedt, O. F.** *With the technical assistance of* **Gosselin, S. M.** Studies on the preservation of blood. IV. The influence of adenosine on the glycolytic activity of the erythrocyte during storage at 4° C., 61.

**Rubinstein, D., Ottolenghi, P.,** and **Denstedt, O. F.** The metabolism of the erythrocyte. XIII. Enzyme activity in the reticulocyte, 222.

**Saffran, M.** See Schönbaum, E., 527.

**Sallans, H. R.** See Spencer, J. F. T., 495.

**Scholefield, P. G.** Studies of fatty acid oxidation. 4. The effects of fatty acids on the oxidation of other metabolites, 1211.

Studies of fatty acid oxidation. 5. The effect of decanoic acid on oxidative phosphorylation, 1227.

**Schönbaum, E., Birmingham, M. K.,** and **Saffran, M.** *With the technical assistance of* **Kurlents, E.** Metabolism of glucose and steroid formation by rat adrenals *in vitro*, 527.

**Seegers, W. H.** See Penner, J. A., 1199.

**Seegers, W. H., Abe, T.,** and **Fenichel, R. L.** Electrophoresis of autoprothrombin and biothrombin, 270.

**Seegers, W. H., Johnson, S. A.,** and **Penner, J. A.** Quantitative concepts related to prothrombin and autoprothrombin I activity, 887.

**Seguin, J. J.** See Drake, C. G., 689.

**Sellers, E. A.** See Baker, D. G., 835.

**Skelton, F. R.** Studies of certain antihistamines as antagonists of the hypertensive and and angiotoxic properties of desoxycorticosterone acetate in the rat, 520.

**Smallman, B. N.** See Chefurka, W., 731.

**Smith, D. B.** See Wood, G. C., 1107.

**Solvonuk, P. F., McRae, S. C.,** and **Collier, H. B.** Creatine phosphokinase activity of mammalian erythrocytes, 481.

**Sourkes, T. L.** See Lagnado, J. R., 1095, 1185.

**Southcott, C. M., Bandy, H. E., Newsom, S. E.,** and **Darrach, M.** Metabolism of adrenal steroids in the mouse. 1. Observations on 20$\alpha$-dihydrocorticosterone and corticosterone in the plasma of mice treated with corticotropin, 913.

**Southcott, C. M., Gandossi, S. K., Barker, A. D., Bandy, H. E., McIntosh, H.,** and **Darrach, M.** Studies on human adrenal steroids. 1. The effect of corticotropin on components of the free and conjugated plasma $C_{21}$ adrenal steroid fractions, 146.

**Spencer, J. F. T., Neish, A. C., Blackwood, A. C.,** and **Sallans, H. R.** Polyhydric alcohol production by osmophilic yeasts: Studies with $C^{14}$-labeled glucose, 495.

**Spencer, M. S.** Ethylene metabolism in tomato fruit. I. Relationship of ethylene evolution to fruit respiration and ripening, 1261.

**Spinks, J. W. T.** See Taylor, J. D., 1143.

**Spoerel, W. E. G. A.** and **Gowdey, C. W.** Cardiovascular effects of pentolinium bitartrate in dogs, 747.

**Staron, N.** See Kalow, W., 637.

**Stavraky, G. W.** See Drake, C. G., 689.

**Stephenson, N. R.** The relative potency of adrenal corticoids by the thymus involution method, 253.

**Stevenson, J. A. F.** See Gowdey, C. W., 951; Rixon, R. H., 1069.

**Steyermark, P. R.** See Nowaczynski, W. J., 592, 1023.

**Szerb, J. C.** See Moya, F., 563.

**Tamboline, B., Matheson, A. T.,** and **Zbarsky, S. H.** Glucosuria in rats receiving British anti-lewisite (BAL), 815.

**Taylor, J. D., Millar, G. J., Jaques, L. B.,** and **Spinks, J. W. T.** The distribution of administered vitamin $K_1$-$C^{14}$ in rats, 1143.

**Thorn, J. A.** See Boothroyd, B., 10.

**Tikkala, A. O.** See Boyd, E. M., 259.

**Towers, G. H. N.** See Wickson, M. E., 502.

**Towers, G. H. N.** and **Mortimer, D. C.** The role of keto acids in photosynthetic carbon dioxide assimilation, 511.

**Triantaphyllopoulos, D. C.** Blood globulins reducing the anticoagulant activity of heparin, 939.

**Trick, G. S.** See Adie, P. A., 80.

**Truscott, B.** and **Hoogland, P. L.** The constituents of cod liver with vitamin $B_{12}$ activity for *Lactobacillus leichmannii*, 191.

**Tuba, J.** See Kovacs, L., 6.

**Turpin, J. E. H.** See Friedman, S. M., 158.

**Vaisey, E. B.** The non-enzymic reduction of trimethylamine oxide to trimethylamine, dimethylamine, and formaldehyde, 1085.

**Waud, D. R.** See Waud, R. A., 827.

**Waud, R. A.** and **Waud, D. R.** Immediate effects of rapid hemorrhage on hemodynamics in the dog, 827.

**Waygood, E. R.** See Maclachlan, G. A., 1233.

**Webber, R. V.** and **Bayley, S. T.** Some observations on the molecular form of chondroitin sulphate, 993.

**Webber, W. A.** See Friedman, S. M., 475.

**Weber, G.** See Allard, C., 170.

**Whitaker, D. R.** The steric factor in the hydrolysis of $\beta$-1,4′-oligoglucosides by *Myrothecium* cellulase, 102.

The mechanism of degradation of a cellodextrin by *Myrothecium* cellulase, 488.

**Whitaker, D. R.** and **Merler, E.** Cleavage of cellotriose by *Myrothecium* cellulase, 83.

**Wickson, M. E.** and **Towers, G. H. N.** $C^{14}O_2$ assimilation in *Lilium regale* with reference to $\gamma$-methyleneglutamic acid, 502.

**Wood, G. C.** and **Smith, D. B.** Size, stability, and heterogeneity of apurinic acid, 1107.

**Wood, J. D.** and **Migicovsky, B. B.** Fatty acid inhibition of cholesterol synthesis, 861.

**Wright, L. A., Maw, W. A.,** and **Common, R. H.** The influence of thiouracil on the responses of serum protein and serum calcium of the immature pullet to estrogen, with some observations on the responses of oviduct, liver, and kidney, 817.

**Young, R. J., Lucas, C. C., Patterson, J. M.,** and **Best, C. H.** Lipotropic dose–response studies in rats: Comparisons of choline, betaine, and methionine, 713.

**Zbarsky, S. H.** See Tamboline, B., 815.

## CORRECTION

Vol. 33, p. 888. The following tabular material should be substituted below the heading of Table I.

| Day of study | 8 a.m. | 12 noon | 3.30 p.m. |
|---|---|---|---|
| 11 | .63 | .61 | — |
| 12* | .51 | .26 | .50 |
| 13 | .43 | .33 | .40 |
| 14 | .47 | .48 | — |

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References should be listed **alphabetically by authors' names,** numbered, and typed after the text. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should not be given and initial page numbers only are required. The names of periodicals should be abbreviated in the form given in the most recent *List of Periodicals Abstracted by Chemical Abstracts*. All citations should be checked with the original articles, and each one referred to in the text by the key number.

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## *Contents*


Page

The Non-enzymic Reduction of Trimethylamine Oxide to Trimethylamine, Dimethylamine, and Formaldehyde—*E. B. Vaisey* - - - - - - 1085

The Purification of Sarinase from Bovine Plasma—*P. A. Adie* - - - - 1091

The Enzymatic Reduction of Tetrazolium Salts by Amines—*J. R. Lagnado and T. L. Sourkes* - - - - - - - - - - - - - - 1095

Size, Stability, and Heterogeneity of Apurinic Acid—*G. C. Wood and David B. Smith* - - - - - - - - - - - - - - - - 1107

The Effect of Aureomycin on the Production of Hemorrhagic Shock in the Rat—*H. G. Downie* - - - - - - - - - - - - - - 1119

The Formation of 2-Pyrrolecarboxylic Acid from Hydroxy-D- and Allohydroxy-D-Proline—*G. Letellier and L. P. Bouthillier* - - - - - - - - 1123

Activation of Schradan by Mammalian Tissue Homogenates—*R. D. O'Brien* - 1131

The Distribution of Administered Vitamin $K_1$-$C^{14}$ in Rats—*J. D. Taylor, G. J. Millar, L. B. Jaques, and J. W. T. Spinks* - - - - - - - 1143

Experiments on the Use of Silicic Acid for Steroid Chromatography—*J. H. Linford* - - - - - - - - - - - - - - - 1153

Enzymatic Properties of Bovine Plasmin Preparations; Evidence for Similarity to but Non-identity with Trypsin—*Edward Ronwin* - - - - - 1169

Inhibition of Amine Oxidase by Metal Ions and Sulphydryl Compounds—*J. R. Lagnado and T. L. Sourkes* - - - - - - - - - - - 1185

Extracellular Organic Nitrogen in *Ustilago maydis* Fermentation Broths—*Eugene L. Dulaney, E. Bilinski, and W. B. McConnell* - - - - - - 1195

Conversion of Prothrombin to Autoprothrombin II—*John A. Penner, F. Duckert, Shirley A. Johnson, and Walter H. Seegers* - - - - - - - - - 1199

Studies of Fatty Acid Oxidation. 4. The Effects of Fatty Acids on the Oxidation of Other Metabolites—*P. G. Scholefield* - - - - - - 1211

Studies of Fatty Acid Oxidation. 5. The Effect of Decanoic Acid on Oxidative Phosphorylation—*P. G. Scholefield* - - - - - - - 1227

Kinetics of the Enzymically-catalyzed Oxidation of Indoleacetic Acid—*G. A. Maclachlan and E. R. Waygood* - - - - - - - - - - 1233

Further Studies on Thyroid – Adrenal – Ascorbic Acid Relations in Animals Exposed to Cold—*André DesMarais* - - - - - - - - - - 1251

Ethylene Metabolism in Tomato Fruit. I. Relationship of Ethylene Evolution to Fruit Respiration and Ripening—*Mary S. Spencer* - - 1261

Notes:

A Mannan Produced by *Bacillus polymyxa*—*D. Murphy, C. T. Bishop, and G. A. Adams* - - - - - - - - - - - - - - - 1271

Contents of Volume 34 - - - - - - - - - - - - - - - 1275